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Impact of Incremental Sampling Methodology (ISM) on Metals Bioavailability

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Impact of Incremental Sampling Methodology (ISM) on Metals Bioavailability

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Abstract

This study assessed the impact of the incremental sampling methodology (ISM) on metals bioavailability through a series of digestion and in vivo experiments. These tests used *Eisenia fetida* and *Lolium rigidum* in both milled and unmilled loam and sand soil containing antimony, copper, lead, and zinc obtained from Donnelly Training Area, Alaska. No significant differences in metal levels were evident between milled and unmilled soil for *E. fetida*, and uptake of lead by *L. rigidum* in sand yielded lead recoveries comparable with Method 3050 analysis of soil. In contrast, *L. rigidum* grown in loam had much lower recoverable lead. Milling of the soil as part of the ISM process had no significant impact on the lead species distribution. In comparison with Method 3050, the alternative digestion tests involving the use of glycine; oxalate; ethylenediaminetetraacetic acid (EDTA); or alternative digestion procedures, such as the synthetic precipitation leaching procedure (SPLP) and the toxicity characteristic leaching procedure (TCLP), yielded lower recoveries of lead for all soil particle sizes and soil types. Diffusive gradient in thin films experiments yielded metal concentrations positively correlated with *E. fetida* concentrations. The physiologically based extraction technique (PBET) positively correlated with bulk soil concentrations and *E. fetida* tissue concentrations for all soils evaluated.

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Preface

This study was conducted for the U.S. Army Environmental Command (AEC) under Project 404632, "Metal Bioavailability Assessment." The technical monitors were Drs. Doris Anders and Robert Kirgan with AEC.

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COL Bryan S. Green was the Commander of ERDC, and Dr. Jeffery P. Holland was the Director.

Acronyms and Abbreviations

ABA	Absolute Bioavailability
AEC	U.S. Army Environmental Command
AFO	Oral Absorption Fraction
Al	Aluminum
As	Arsenic
ASTM	American Society of Testing Methods
Ba	Barium
Ca	Calcium
$CaCO_3$	Calcium Carbonate
Cr	Chromium
CRREL	Cold Regions Research and Engineering Laboratory
Cu	Copper
DGT	Diffusive Gradients in Thin Films
DI	Deionized
DOD	U.S. Department of Defense
DU	Decision Units
EC50s	Half Maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
EL	Environmental Laboratory
ERDC	Engineer Research and Development Center
ESTCP	Environmental Security and Technology Certification Program
Fe	Iron

HCl	Hydrochloric Acid
HDPE	High-Density Polyethylene
HNO_3	Nitric Acid
ICP-MS	Inductively Coupled Plasma–Mass Spectrometry
ICP-OES	Inductively Coupled Plasma–Optical Emission Spectroscopy
ISM	Incremental Sampling Methodology
ITRC	Interstate Technology Regulatory Council
K	Potassium
LC50s	Half Maximal Lethal Concentration
Mg	Magnesium
Mn	Manganese
MS	Mass Spectroscopy
Na	Sodium
Ni	Nickel
NIST	National Institute of Standards and Tests
NRC	National Resource Council
NT	Not Tested
OLS	Ordinary Least Squares
P	Phosphorus
Pb	Lead
Pb(II)	Exchangeable Lead
Pb^{2+}	Residual Lead
PBET	Physiologically Based Extraction Technique
PbC	Organic Lead

$PbCO_3$	Lead Carbonate
PbO	Lead Oxide
PbS	Lead Sulfide
PbSol	Soluble Lead
RBA	Relative Bioavailability
Sb	Antimony
SD	Standard Deviation
Si	Silicon
SPAWAR	Space and Naval Warfare Systems Command
SPLP	Synthetic Precipitation Leaching Procedure
SRM	Standard Reference Material
TCLP	Toxicity Characteristic Leaching Procedure
TMG	Trace-Metal Grade
USACE	U.S. Army Corps of Engineers
USEPA	U.S. Environmental Protection Agency
V	Vanadium
WDOE	Washington State Department of Ecology
Zn	Zinc

1 Introduction

1.1 Background

The U.S. Environmental Protection Agency (USEPA) has adopted the incremental sampling methodology (ISM) as the accepted method (Method 8330B, 8330C) for sample collection and processing of soils containing energetic residues on U.S. Department of Defense (DOD) training and testing ranges (USEPA 2014, 2006a; Hewitt et al. 2009; Walsh et al. 2005; Jenkins et al. 2004, 2005; and Pitard 1993). In addition to energetics, incremental sampling and associated processing procedures are increasingly being adopted for other constituents introduced in particulate form, such as metals (Hewitt et al. 2011, 2009; ITRC 2012; Alaska 2009; Hawaii 2008). ISM is a

> *structured composite sampling and processing protocol that reduces data variability and provides a reasonably unbiased estimate of mean contaminant concentrations in a volume of soil targeted for sampling. ISM provides representative samples of specific soil volumes defined as decision units (DUs) by collecting numerous increments of soil (typically 30–100 increments) that are combined, processed, and sub-sampled according to specific protocols* (ITRC 2012).

Initially, ISM is focused on correct field sampling, then various manipulations of the samples are performed to create a single homogenized sample that is analyzed for the constituents of interest, providing a more representative average concentration of the selected study area.

The Environmental Security and Technology Certification Program (ESTCP) funded ER-0918 project, which developed new sampling and sample preparation procedures falling under the ISM umbrella for soils containing metal particulates (Clausen et al. 2012, 2013a, 2013b, 2013c). USEPA Method 3050C will be introduced in the Method VI update to SW-846 in 2016 (USEPA, forthcoming). However, the impact on sample processing, principally machining of the sample to reduce particle size, and its effect on metal bioavailability and ultimately human and ecological risk is

unknown (Clausen 2015). The ISM protocols may introduce a positive bias in extraction efficiencies and bioavailability; the multi-increment sampling methodology dictates that samples be ground to a particle size of 75 µm to achieve a fundamental error of less than 15% (Hewitt et al. 2009). The act of milling to such a fine particle size may increase the exposure and bioavailability of contaminants to test organisms used in toxicological bioassays.

The DOD has established directives mandating that all DOD facilities implement procedures to assess environmental impacts of munitions on training and testing ranges (DOD 2004, 2005). Presently, many DOD installations are being directed to implement changes to their sample and sample processing of soil and sediment samples for metals (Alaska 2009; Hawaii 2008) in the absence of data showing that these changes are appropriate for assessing human and ecological risk and for establishing soil cleanup levels. However, a common approach for calculating risk associated with soil exposure is by collecting and analyzing soil by using USEPA Method 3050B (USEPA 1996) for digestion followed by Methods 6010 and 6020 for analysis (USEPA 2006b, 2006c). Method 3050B states

> *This method is not a total digestion technique for most samples. It is a very strong acid digestion that will dissolve almost all elements that could become "environmentally available*."

Unfortunately, Method 3050B (USEPA 1996) does not define what is meant by "environmentally available" and whether this is equivalent to bioavailability. Within the environmental industry, there is a lack of consensus on the proper sample preparation and analysis methods for soils containing metallic residues at military ranges. Studies with different physiologically based bioavailable extraction tests yield different results. The document *Bioavailability of Contaminants in Soils and Sediments: Processes, Tools, and Applications* (NRC 2003) states the following:

> *Replacing default values with site-specific information should be encouraged. . . . There is no clear regulatory guidance or scientific consensus about the level and lines of evidence needed for comprehensive bioavailability process assessment.*

Therefore, the U.S. Army Environmental Command (AEC) funded this project to address a U.S. Army concern on whether the widespread adoption of ISM as part of USEPA Method 3050B update would lead to a bias in metals bioavailability results. Specifically, the concern relates to the milling step of the ISM process and the assumption that this activity would result in elevated metals levels as compared to the conventional sample processing approach.

1.2 Objectives

The three objectives for this study were (1) to determine whether incorporating sample processing changes similar to those in Method 8330B into Method 3050B yield soil or sediment metal concentrations appropriate for human and ecological risk assessment; (2) to identify the appropriate bioavailability tests for various metals, depending on the different soil and sediment types for ranges and to establish the relationship with Method 3050C; and (3) to determine whether the oversize fraction, >2 mm in size, can be ignored as USEPA does not consider this material to be soil. Further, this study proposes providing context for the modified USEPA method for metals in relation to bioavailability assessment approaches. Our hypothesis is that milling (sometimes referred to as grinding) of soil will change the estimated bioavailability of a particular metal; that metal bioavailability is dependent on soil type, which has bearing on the appropriateness of a given bioavailability test; and that the oversize fraction contains a significant metal mass that should not be ignored.

1.3 Approach

Our study approach involves standard soil toxicity tests and novel techniques conducted in several phases. The in vitro studies focused first on the development of toxicity metrics (e.g., half maximal effective concentrations [EC50s] and half maximal lethal concentrations [LC50s]) for the common lumbriculid worm, *Eisenia fetida*, in soils spiked with copper using standardized protocols (ASTM 1997; WDOE 1996). Second, our study tested both uncontaminated and contaminated soils having undergone grain size partitioning prior to testing (as part of the ISM protocol). The study used the earthworm (*Eisenia fetida*) for toxicology and bioaccumulation bioassays (ASTM 2009) and the ryegrass (*Lolium rigidum*) for a seed germination bioassay (ASTM 2004). Various digestion experiments (Clausen et al. 2010; USEPA 2007; Rodriguez et al. 1999; Ruby et al. 1996,

1999) were conducted to assess the relative bioavailability (RBA) of metals in soil or soil-like samples by measuring the rate and extent of metal solubilization in an extraction solvent that resembles gastrointestinal fluids. The fraction of metal that solubilizes in an in vitro system is referred to as in vitro *bioaccessibility*. This method may provide a faster and less costly alternative for estimating RBA of metals than in vivo methods.

2 Incremental Sampling Methodology

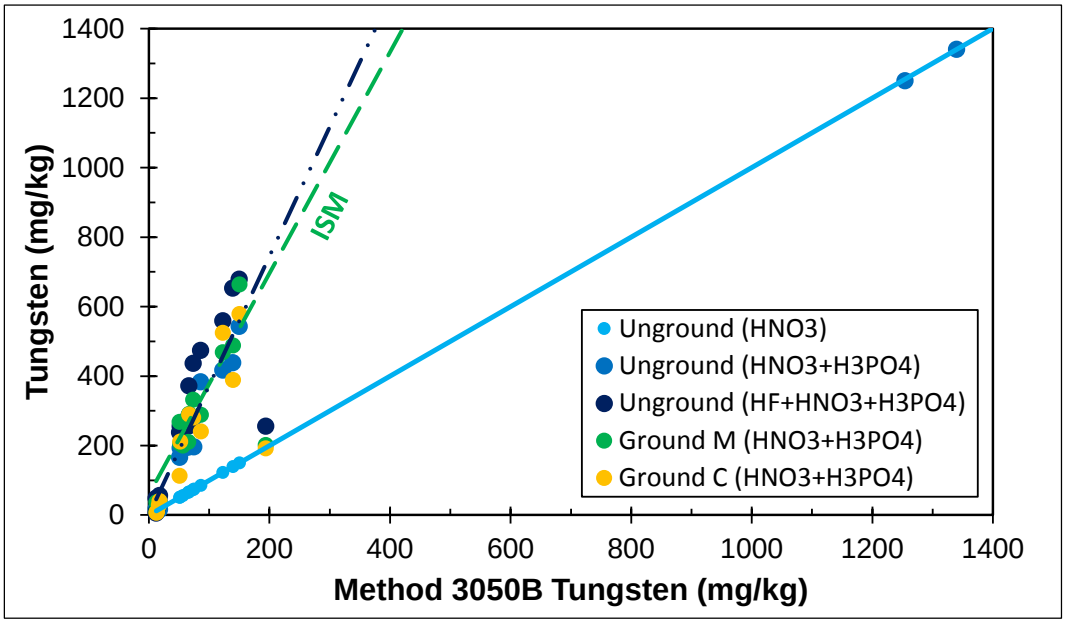
Multi-increment sampling has been established as the proper methodology for evaluating particulate deposition of energetic residues (Hewitt et al. 2009; Ramsey and Hewitt 2005; Walsh et al. 2005; Jenkins et al. 2005, 2004; and Pitard 1993) and has been adopted as a new USEPA Method 8330B (USEPA 2006a) and 3050C (USEPA, forthcoming). There has been increasing push to adopt the multi-increment sampling methodology for other analytes, including metals (Hewitt et al. 2011; ITRC 2012; Alaska 2009; Hawaii 2008). One sample processing step of the multi-increment sampling methodology involves machining (or grinding) of the sample to increase the number of particulate contaminants of interest present in the sample. ISM dictates that the samples be ground to a particle size less than 75 µm to achieve a fundamental error of less than 15% (Hewitt et al. 2009).

The act of grinding to such a fine particle size may increase the exposure and bioavailability of contaminants to test organisms used in toxicological bioassays; a topic explored in this study. Bioavailability studies are commonly used to assess the toxicity and bioavailability of a particular metal to human and ecological receptors.

Another issue relates to the standard USEPA Method 3050B used for metals digestion (USEPA 1996), which according to the method yields the environmental fraction that a human or ecological receptor may encounter. However, there is no documentation in the literature that establishes the relationship between this environmental available fraction and accepted bioavailability tests. Consequently, it is not possible to place the Method 3050B value in the proper context in regards to bioavailability. Our earlier work evaluating different digestion procedures for tungsten in soil (Clausen et al. 2010) indicated Method 3050B recovered considerably less tungsten than did some commonly used European Union digestion methods (Figure 1). In addition, a modified Method 3050B involving milling and some other changes to the sample preparation methods (Clausen et al. 2012) yielded results closer to presumed total digestion methods. Yet, the results from using Method 3050B are typically used by risk assessors to compute the human and ecological risk or to compare against soil reme-

dial action levels. The present study will identify the appropriate bioavailability test for metals and will establish the relationship between Method 3050C and Method 3050B.

Figure 1. Comparison of prior digestion results for tungsten.



The U.S. Army questioned whether the subsequent reduction in soil and contaminant particle size through milling to control subsampling analytical errors might alter the relationship between the concentration of metals reported and their actual bioavailability as compared to the unground or conventionally prepared soil or sediment sample. Such an effect would have a significant impact on inferences of human and ecological risk when using Method 3050. Because metals in soils are found in a variety of mineral associations and chemical combinations of varying stability or solubility, the total metal content of a soil or sediment based on Method 3050B often does not correlate well with toxicity or bioavailability measures due to differences in digestion efficiencies (Rodriguez et al. 1999; Ruby et al. 1999, 1996). The bioavailable metal is typically only a fraction of the total metal content that is truly available and capable of producing a toxic response. Despite this fact, risk assessors often use Method 3050 digestion procedure to determine human or ecological risk or to set soil remedial action levels. If Method 3050 is to be used as an index of that risk, the relationship between toxicity/bioavailability and the analytical concentrations reported by the modified 3050 method must be understood.

A term often used when discussing bioavailability is *absolute bioavailability* (ABA), which is the ratio of the amount of metal absorbed compared to the amount ingested, also referred to the oral absorption fraction (AFO):

$$\text{ABA} = \text{AFO} / \text{Ingested Dose} \quad (1)$$

For example, if 100 micrograms (μg) of lead dissolved in drinking water were ingested and a total of 50 μg entered the body, the ABA would be 50/100 or 0.50 (50%). Likewise, if 100 μg of lead contained in soil were ingested and 30 μg entered the body, the ABA for soil would be 30/100 or 0.30 (30%). If the lead dissolved in water were used as the frame of reference for describing the relative amount of lead absorbed from soil, the RBA would be 0.30/0.50 or 0.60 (60%).

$$\text{RBA} = (|\text{ABA}| \times \text{test material}) / (|\text{ABA}| \times \text{reference material}) \quad (2)$$

RBA is the ratio of the absolute bioavailability of a metal present in some test material compared to the absolute bioavailability of the metal in some appropriate reference material.

3 Methods

Our study used a variety of methods to determine the amount of metal present in the two soils tested and included the following:

- Digestion methods using nitric acid (HNO_3) (Method 3050C and B), oxalate, glycine, ethylenediaminetetraacetic acid (EDTA), synthetic precipitation leaching procedure (SPLP), toxicity characteristic leaching procedure (TCLP), and sequential digestion (Tessier et al. 1979)
- In vitro bioaccessibility (Drexler and Brattin 2007)
- Varying particle sizes (sieving and grinding)
- In vivo survival and bioaccumulation studies over 14 and 28 days in the earthworm (*Eisenia fetida*)
- In vivo survival and bioaccumulation studies over 8 months in the ryegrass (*Lolium rigidum*)
- Physiological based extraction technique (PBET)
- Diffusive gradients in thin films (DGT)
- Analysis with inductively coupled plasma–optical emission spectroscopy (ICP-OES) and ICP–mass spectroscopy (MS) (Methods 6010/6020)

The earthworm (*E. fetida*) was used for toxicology and bioaccumulation bioassays (ASTM 2009), and ryegrass (*L. rigidum*) was used for a seed germination bioassay (ASTM 2004). The study evaluated in vitro bioaccessibility by using the method of Drexler and Brattin (2007), which the USEPA has approved for lead. Initial particle size testing looked at any effects the milling process alone had on both plant and invertebrate bioassays. The smaller particle size itself may be toxic and influence the results of the bioassays without any related contaminant toxicity. Clean artificial control soil was made based on the formula outlined in American Society of Testing Methods (ASTM) Methods E1676-04 and E1963-09 for the earthworm and ryegrass, respectively (ASTM 2009, 2004). Our study performed a series of toxicity tests on a control (unmilled) soil and on a series of processed soil milled to different particle sizes (e.g., <2 mm to 250 µm and <250 µm). A split of these same samples was analyzed using the USEPA Method 3050B. The results of this set of experiments guided the

particle sizes used for the site soil testing and allowed a point of comparison between the total metal content of the soil and the environmentally available metal as ascertained by Method 3050C.

3.1 Field sampling

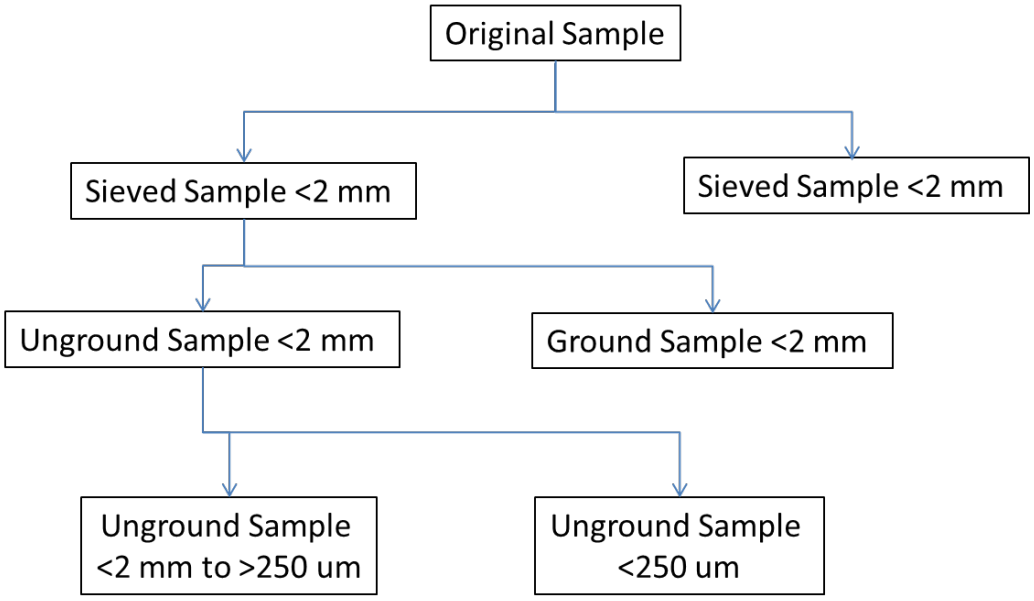
Soil samples were collected on 2 October 2013 from the Texas small-arms range berms (Figure 2) at the Donnelly Test Area, AK, where 200 rounds of 7.62 mm ammunition were fired with an M-16 rifle. A total of 50 increments were collected from each berm following ISM (Clausen et al. 2013b, 2012; ITRC 2012). Soil contamination consisted of the metals antimony, copper, lead, and zinc. The contaminated berms sampled were constructed of loam and sand. Uncontaminated control berms of each material were also sampled for a total of four site samples. Samples were collected using the multi-increment sampling methodology sampling guidelines, Method 3050C, with a minimum of 50 increments used to create one single sample. To accurately address variability, each of the four berms was sampled in triplicate, resulting in three replicate samples (each consisting of 50 increments).

Figure 2. Collection of field samples from the small-arms range berm at the Texas Range on the Donnelly Training Area, AK.

3.2 Laboratory sample preparation

Once the field samples were collected, they were shipped back to the ERDC Cold Regions Research and Engineering Laboratory (CRREL) in Hanover, NH, for laboratory preparation. The samples were air dried and sieved with a No. 10 mesh sieve to remove the >2 mm fraction, which is commonly discarded (Figure 3). The USEPA does not consider >2 mm to be soil even though this fraction can be a sizeable portion of the total metal mass. The <2 mm fraction was then split in half with a Lab Tech Essa sectorial rotary splitter (Model RSD 5/8, Belmont, Australia) operated at 100 rpm. The weight for both splits was recorded. One of the <2 mm splits was used for the unmilled experiments and the other for the milled experiments.

Figure 3. Study design sample processing hierarchy.



The ground fraction was created using the ISM techniques, which involved using a Lab Tech Essa chrome steel ring mill grinder (Model LM2, Belmont, Australia) for five 60 sec intervals with 60 sec of cooling between each interval. This length of grinding typically yields a material size less than 150 µm (Hewitt et al. 2009).

The unground <2 mm sample was sieved with a no. 60 sieve, yielding >250 µm and <250 µm fractions. The <250 µm fraction can stick to the

hand due to electrostatic forces. Therefore, some risk assessors require that analysis of this material yields a conservative risk calculation.

Each soil sample yielded 7 subsamples with 2 contaminated soils (loam and sand) and two controls (loam and sand) for a total of 28 subsamples. This material was then digested using a variety of extractants and methods.

3.3 Soil characterization

Solid samples were digested according to USEPA Method 3050B using nitric acid and hydrogen peroxide. Hydrochloric acid was not used to reduce matrix interferences from chloride ions in the subsequent ICP-MS analyses. In certain cases, such as with plant tissues, additional hydrogen peroxide was used above the 10 mL described in the method if required to destroy residual organic matter prior to filtration, dilution, and analysis.

A series of sequential extractions was also performed to determine the speciation for lead (Baumann and Fisher 2011; Tessier et al. 1979). The most bioavailable metals fraction is the labile fraction, which is loosely associated with soil particles. This labile fraction is easily extractable with magnesium chloride and sodium acetate at pH 5. Magnesium chloride yields what is referred to as the exchangeable lead. Sodium acetate recovers lead species associated with carbon, and the soluble fraction of lead is obtained using deionized (DI) water. Hydroxylamine hydrochloride is used to recover lead oxides; and a mixture of hydrogen peroxide, nitric acid, and hydrochloric acid is used to recover lead species associated with organic matter and sulfides. Any remaining lead after the sequential digestion is referred to as the residual lead, which tends to be the insoluble solid lead species. The sequential metal extraction process allows for a better discrimination of the influence of milling on the bioavailable fractions of the metals versus total metal concentrations alone.

Glycine was used as an extractant following the procedures in USEPA (2007). The glycine procedure is supposed to simulate a synthetic gastric juice and has been previously validated using in vivo juvenile swine tests (Drexler and Brattin 2007). An extraction using disodium EDTA at pH 7.0 was performed following the method of Quevauviller et al. (1997). This reagent sequesters metal ions associated with calcium (Ca^{2+}) and iron (Fe^{3+}),

thus solubilizing the metals, allowing for aqueous analysis. An acidic oxalate extraction was performed to solubilize metals bound to iron sulfides (Chen et al. 2013). In addition, other digestions included SPLP Method 1312 (USEPA 1994) and TCLP Method 1311 (USEPA 2008).

3.4 In vitro experiments

In vitro bioaccessibility was evaluated using the method of Drexler and Brattin (2007). Worm tissues were analyzed for a suite of metals at the culmination of the bioaccumulation tests. Supporting chemical analysis on the four soil subsamples was performed to assess initial metal concentration (see Section 3.3).

3.4.1 Organism procurement and handling

The test organism used in this study was the earthworm, *E. fetida*. Cultured *E. fetida* were obtained from Uncle Jim's Worm Farm in Spring Gove, PA. Adult (based on size) *E. fetida* arrived via overnight delivery to the Space and Naval Warfare Systems Command, Bioassay Laboratory, San Diego, CA. On arrival at the laboratory, organism receipt information was recorded and animal condition was noted. All test organisms were held at 23 ± 1°C until testing was initiated. During the acclimation period, the animals were observed for any indications of stress or significant mortality, which was recorded in organism holding logbooks.

3.4.2 Test material

Phase I test material consisted of laboratory-prepared artificial soil and clean beach sand. The artificial soil was prepared using ASTM guidance (ASTM 2009) and was a combination of 70% beach sand, 20% kaolin clay, 10% peat moss, and 0.4% calcium carbonate ($CaCO_3$). All ingredients (with the exception of $CaCO_3$) were washed, dried, and sieved prior to preparation. Artificial soil was aged for two weeks prior to use and adjusted with $CaCO_3$ to pH 7.0. Artificial soil was mixed in varying ratios with beach sand to assess if there would be an adverse effect on the test organisms due to the amount of sand in the mixture. Each artificial soil and sand mixture was then spiked to varying concentrations of copper to see if there was an interactive effect of grain size distribution on the bioavailability of copper to the organisms. Table 1 shows the treatments that Phase I tested.

Table 1. Artificial soil mixtures and treatments.

Treatment	Copper Spiking Concentrations
100% Sand	0, 50, 100, 200 ppm
25%/75% Sand / Artificial Soil	0, 50, 100, 200 ppm
50%/50% Sand / Artificial Soil	0, 50, 100, 200 ppm
100% Artificial Soil	None

Phase II test material consisted of field-collected soil samples, discussed in Section 3.1, that underwent grain size partitioning and the ISM protocol. All site samples were mixed on a 50:50 basis with artificial soil to reduce the potential of earthworm mortality due to the grain size distributions of the site soils. Table 2 indicates the site samples that were tested upon receipt in Phase II along with their respective soil characteristics. Subsamples of each soil were taken for metals analysis using ICP-MS (Method 6020) following digestion using Method 3050B.

Table 2. Field-collected soils.

Sample ID	Sample Description	Grain Size Fractionation	Moisture Content Determination upon Receipt (%)	Water Holding Capacity (%)
BS-1B	Background Sand	Sieved >2 mm	1.77	36
BS-1AUa	Background Sand	Sieved >250 µm to <2 mm	1.66	43
BS-1AUb	Background Sand	Sieved <250 µm	2.40	53
BS-1AG	Background Sand	Ground <2 mm	2.39	55
BL-1B	Background Loam	Sieved >2 mm	2.94	49
BL-1AUa	Background Loam	Sieved >250 µm to <2 mm	2.48	64
BL-1AUb	Background Loam	Sieved <250 µm	2.02	57
BL-1AG	Background Loam	Ground <2 mm	3.04	58
CS-1B	Contaminated Sand	Sieved >2 mm	2.12	38
CS-1AUa	Contaminated Sand	Sieved >250 µm to <2 mm	1.96	41
CS-1AUb	Contaminated Sand	Sieved <250 µm	2.12	45
CS-1AG	Contaminated Sand	Ground <2 mm	1.96	47
CL-1B	Contaminated Loam	Sieved >2 mm	2.98	45
CL-1AUa*	Contaminated Loam	Sieved >250 µm to <2 mm	-	-
CL-1AUb	Contaminated Loam	Sieved <250 µm	3.15	64
CL-1AG	Contaminated Loam	Ground <2 mm	2.94	52

*Soil sample not received at the SSC Bioassay Lab.

3.4.3 Earthworm survival, growth, and bioaccumulation test

Earthworm bioassays (Figure 4) were conducted in accordance with ASTM (1997) and the Washington State Department of Ecology (WDOE 1996). A summary of test conditions for the earthworm survival, growth, and bioaccumulation tests is contained in Table 3 and described in detail in the following section.

Figure 4. Earthworm experimental layout.

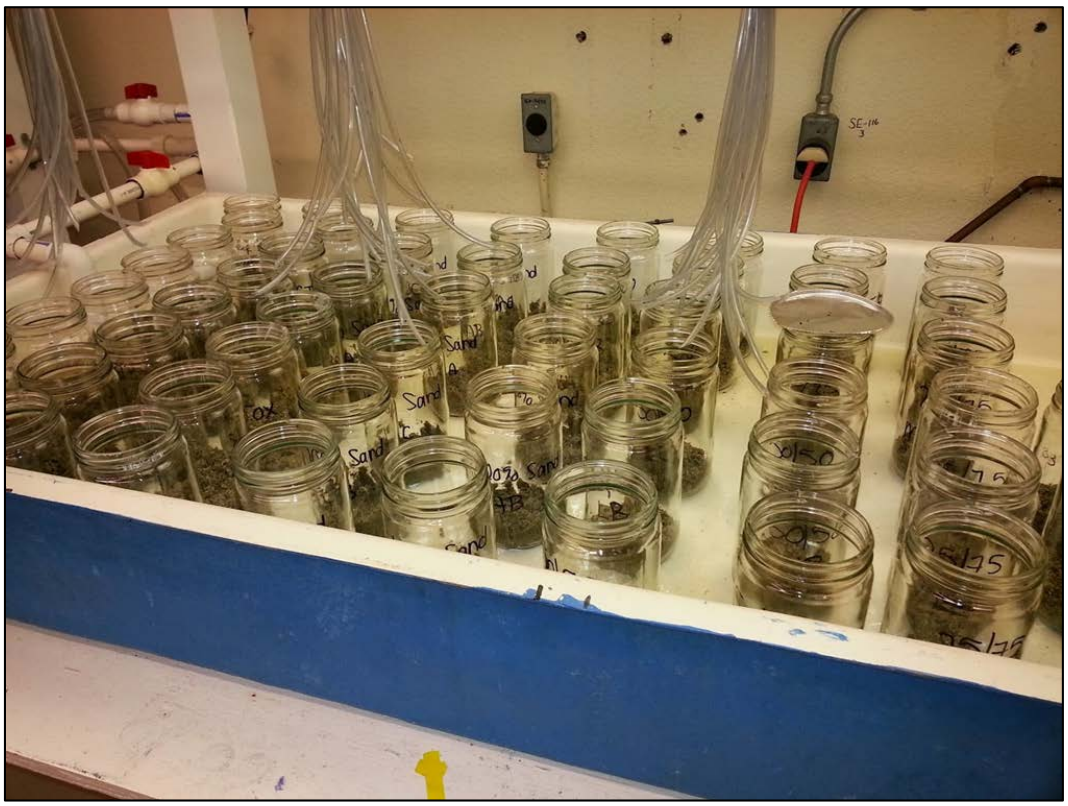


Table 3. Earthworm toxicity and bioaccumulation test specifications.

Test periods	Phase I: 11/5/2013–11/19/2013 (14 days) Phase II: 3/7/2014–4/4/2014 (28 days)
Test endpoints	Survival, Growth, Bioaccumulation
Test organism	*Eisenia fetida* (earthworm)
Test organism source	Uncle Jim's Worm Farm, Spring Grove, PA
Feeding	None
Depuration period	22–24 hr
Test chamber	1 L glass jar
Test solution volume	Approximately 200 g per replicate
Number of organisms/chamber	10
Number of replicates	4
Hydration water	Deionized water
Additional control	Artificial soil
Test temperature	23 ± 1°C
Photoperiod	Continuous light
Test Protocol	ASTM 1997, WDOE 1996
Test acceptability criteria for controls	Mean control survival ≥90%; test organisms should burrow in test soils; instantaneous temperature maintained between 20°C and 26°C; mean test temperature at 23 ± 1°C.
Reference toxicant	2-Chloroacetamide

Earthworms (Figure 5) were exposed to test soils for 14 or 28 days to assess survival, growth, and the potential for bioaccumulation of contaminants from the soil. Test chambers consisted of 1 L glass jars with perforated lids to allow air exchange. The experimental design consisted of four replicate jars per treatment or site. A subsample of sieved test soil (20 g) was set aside for initial moisture fraction determination. Samples were then hydrated to an appropriate moisture level using DI water. Because of the differences in soil composition (texture, structure, and organic content), hydrating soils to a standard level can be problematic. One soil may appear very wet and even have standing water on the surface while another may appear considerably drier after being hydrated to the recommended hydration level of 45% of its dry weight. To address such differences, an approved alternative protocol method was used where an artificial control soil was hydrated to 45% of its dry weight as a standard. All sites then were hydrated to a level approximating the texture and visual appearance of the hydrated artificial soil control.

Figure 5. Earthworms used in the study.

After hydration of test soils, a 20 g subsample was collected for determination of initial soil moisture content and pH (Table 4). The soils were thoroughly homogenized prior to distribution to each replicate chamber. A soil control was conducted concurrently with the test soils by using ASTM artificial soil (described above) to ensure that organisms were not affected by stresses other than contamination in the test material. The control consisted of a formulated soil mixture composed of 70% rinsed beach sand, 20% Kaolin clay, 10% peat moss, and 0.4% $CaCO_3$ by weight. All ingredients (with the exception of $CaCO_3$) were washed with DI water, dried, and sieved prior to preparing soil. The artificial soil then was hydrated to 45% of its dry weight by adding DI water.

Each replicate test chamber received approximately 200 g of control or test soil. The test chambers were placed in an environmental chamber maintained at 23 ± 1°C under a continuous light regime. Soils were allowed to settle and equilibrate for 24 hr prior to the addition of test organisms. Ten earthworms were added to each test chamber after confirmation

that the test organisms were in healthy condition. The worms were not fed during the test period.

Table 4. Initial quality parameters for field-collected soils samples.

Sample ID	Sample Description	Grain Size Fractionation	Moisture Content Determination at Initiation (%)	pH at Initiation
BS-1B	Background Sand	Sieved >2 mm	13.75	7.42
BS-1AUa	Background Sand	Sieved >250 µm to <2 mm	15.56	7.42
BS-1AUb	Background Sand	Sieved <250 µm	18.41	7.48
BS-1AG	Background Sand	Ground <2 mm	19.06	7.50
BL-1B	Background Loam	Sieved >2 mm	12.23	7.35
BL-1AUa	Background Loam	Sieved >250 µm to <2 mm	22.75	7.45
BL-1AUb	Background Loam	Sieved <250 µm	20.00	7.55
BL-1AG	Background Loam	Ground <2 mm	19.29	7.54
CS-1B	Contaminated Sand	Sieved >2 mm	12.51	7.47
CS-1AUa	Contaminated Sand	Sieved >250 µm to <2 mm	10.18	7.34
CS-1AUb	Contaminated Sand	Sieved <250 µm	9.37	7.33
CS-1AG	Contaminated Sand	Ground <2 mm	11.54	7.40
CL-1B	Contaminated Loam	Sieved >2 mm	11.08	7.35
CL-1AUa*	Contaminated Loam	Sieved >250 µm to <2 mm	-	-
CL-1AUb	Contaminated Loam	Sieved <250 µm	16.47	7.32
CL-1AG	Contaminated Loam	Ground <2 mm	16.13	7.38

*Soil sample not received at the SSC Bioassay Lab.

Temperature was monitored daily in the “A” replicate chamber. Abnormal conditions or unusual animal behavior, if observed, were also noted at this time. Examples of unusual behavior include failure to bury, erratic or slow movements, and slow response to stimulation.

Earthworm survival was assessed on both day 14 and at the end of the exposure on day 28. A measure of survival at 14 days was accomplished by emptying the contents of four replicate jars (one at a time) into a clean plastic tray and gently sorting with gloved hands to locate the worms. The number of surviving worms was recorded, and they were placed back in the same replicate jar with soil to continue for the remainder of the 28-day test period. After placing the replicates back into the environmental chamber, all replicates were hydrated with an additional small amount (3–4 mL) of DI water to ensure adequate moisture content for the remainder of the test period.

At the 28-day test termination point, each of the 4 replicates was emptied (one at a time) into a clean plastic tray and gently sorted with gloved hands to locate the worms. The number of surviving worms in each replicate and their composite wet weight were recorded. Dead worms were removed and discarded. The surviving worms were rinsed with DI water to remove any soil and were placed in a clean 500 mL plastic Tupperware with moist paper towels to depurate overnight. The following day, worms were removed from the depuration chambers, weighed again, placed in labeled plastic Ziploc bags, and immediately placed in a freezer for later analysis.

Concurrent 14-day survival reference toxicant tests using 2-chloroacetamide added to control soil were conducted to evaluate the relative sensitivity of the organisms relative to other studies in the literature and to ensure the performance of methods used.

3.4.4 Diffusive gradients in thin films (DGT)

DGT is a relatively new approach to the in situ measurement of metal concentration, flux, bioavailability and speciation in water, sediments, soils, and pore water (Zhang and Davison, 1999, 1995; Harper et al. 1998; Zhang et al. 1998, 1995). The basic soil DGT probe design uses two thin layers composed of a gel layer containing a binding resin such as Chelex 100 and a diffusive hydrogel layer. The theory behind the application is that metals must pass through the diffusive gel layers before contacting and binding to the resin gel layer. The general equation used to calculate the pore water metal concentration is

$$C = \frac{M\Delta g}{DtA} \tag{3}$$

where

Δg = the thickness of the diffusive gel thickness (known),
M = the metal accumulated mass (moles measured),
D = the diffusion coefficient (known),
T = the time for deployment, and
A = the area of the exposed diffusive layer (cm^2).

The ease of deployment of DGTs makes them a suitable tool for assessing the bioavailability of metals. Subsamples of test soils were thoroughly saturated with Milli-Q DI water to create a slurry. DGTs were then firmly placed on top of the slurry for a period of 24 hrs. Upon recovery, DGTs were rinsed well with DI water. At the time of deployment and retrieval, the soil temperature and time was recorded for concentration calculations. To prepare the gel for analysis, the membrane filter and diffusive gel layers were peeled from the probe; and the resin gel layer was removed and rinsed with DI to remove any residual particles or water. The resin layers were then placed in centrifuge tubes and digested with 200 µL of HNO_3. The digestate was then analyzed for metals by ICP-MS Method 6020 (USEPA 2006b).

3.4.5 Physiologically based extraction technique (PBET)

PBET provides an estimate of the RBA of metals in soil or soil-like samples by measuring the rate or extent of metal solubilization in an extraction solvent that resembles gastrointestinal fluids. This technique mimics digestion in the human gut, resulting in a means to understand the human health risk of metals in soils.

Subsamples of test soils were thoroughly dried in an oven at 60°C. Aliquots (1.0 g) of soils were placed in 125 mL HDPE (high density polyethylene) bottles with 100 mL of a prepared glycine solution. The resulting mixture was placed in a pre-warmed water bath and mixed for 1 hr. Following a short period to allow the soil to settle, a 15 mL aliquot of the supernatant was collected and filtered through a 0.45 µm cellulose acetate disk filter (to remove any particulate matter). The filtered samples were then analyzed for metals by ICP-MS (Method 6020).

3.4.6 Metals analysis

Assessment of metal concentrations was made following methodology recommended by the USEPA, including use of trace-metal clean sampling techniques in the collection, handling, and analysis (USEPA 1996). Soil, DGT, tissue digestates, and PBET samples were analyzed using ICP-MS. Three duplicate samples were chosen at random for each run. For every five samples, a blank was run to make sure the system was clean and to give a reference point for the background level of metals. A standard refer-

ence material (SRM) was run after each blank to ensure that the instrument was measuring accurately and precisely. The blank was either 1N trace-metal-grade (TMG) HNO_3 or 18 $M\Omega\ cm^{-1}$ water. The standard was SRM 1643e (trace metals in water) from the National Bureau of Standards. In addition, six blanks were prepared using empty 30 mL HDPE bottles and were treated in the same manner as the soil digestions. All acid additions and dilutions were carried out identically.

3.4.6.1 Soil digestion

Empty 30 mL HDPE bottles were labeled and dried at 60°C in a drying oven for at least 24 hr. The dried bottles were then weighed, and the tare mass (g) recorded. Enough wet sediment to get a dry mass of approximately 0.25 g was transferred to each 30 mL bottle. The bottles (with no caps) were placed in the oven at 60°C for at least 24 hr, followed by verification of complete dryness. The bottles with dry soil were weighed again, and the mass (g) was recorded. One mL of concentrated TMG Hydrochloric Acid (HCl) and 0.5 mL of concentrated TMG HNO_3 were added to each soil sample. The samples were allowed to digest for 24 hr at room temperature, followed by warming on a heating plate (≈60°C) for at least 1 hr. Subsequently, about 30 mL of 1N TMG HNO_3 was added to each sample and the final mass (g) recorded. After particles were allowed to settle, sample dilutions of the overlying digestate were made. For the first phase, a fivefold dilution of each sample was made before metal concentration analysis by transferring 2 mL of sample digestate solution (no particles) to a 15 mL centrifuge tube and adding 8 mL of 1N TMG HNO_3 for a total volume of 10 mL. For the second phase, a tenfold dilution of each sample was made by transferring 1 mL of sample to a centrifuge tube and adding 9 mL of 1N TMG HNO_3 for a total volume of 10 mL.

3.4.6.2 DGT and tissue digestion

Polypropylene microcentrifuge tubes (1.5 mL) were acid cleaned, dried, and weighed. Dry tissue was then placed in the tared tube and dried at 60°C. The DGT gel was set at the bottom of the centrifuge tube and allowed to dry in a class-100 clean bench for several days at room temperature. Once the tissues or the gels were dry, the vials were weighed again and recorded as vial mass plus the DGT or tissue. Concentrated, ultra-pure HNO_3 (50 µL) was added to each vial, making sure to cover the DGT gel film or the tissue as much as possible. The vials were allowed to digest for

at least three days at room temperature in the clean bench. Finally, 1500 µL 1N HNO_3 was added to each vial; and the vial was weighed again.

3.5 Vegetation experiments

The same sample hierarchy used for the in vitro experiments was used for the survival and bioaccumulation study of ryegrass (*Lolium rigidum*) over 8 months (Table 4). There were four soil samples for each type of material: contaminated and uncontaminated loam and contaminated and uncontaminated sand. Each soil had 4 subsamples of different particle size yielding 16 different conditions. Each of the 16 soil variables were tested in quadruplicate (Table 5).

Table 5. Experimental design for the vegetation study.

Sample ID	Rep No.	Bottle Label	Soil Sample Mass (g)	Sample Events Effluent Volume (mL)						
				1	2	3	4	5	6	7
BS-1B	1	1	150.3	300						
BS-1AUa	1	2	150.18	300	275	625	175	50	150	
BS-1AUb	1	3	89.55	300	275	625	225	150		
BS-1AUG	1	4	118.65	300	275	625	175	50	150	
BL-1AUa	1	5	70	300	275	625	175	50	150	
BL-1AUb	1	6	117.35	300	275	625	175	50	150	
BL-1AG	1	7	111.76	300	275	625	175	50	150	
CS-1B	1	8	154.8	300						
CS-1AUa	1	9	150.25	300	275	475	150	175	50	150
CS-1AUb	1	10	69.7	300	225	280	220	180	120	
CS-1AG	1	11	150.09	300	275	625	225	150		
CL-1AUa	1	12	69.23	300	275	625	200	25	150	
CL-1AUb	1	13	114.34	300	275	625	175	50	150	
CL-1AG	1	14	124.28	300	275	625			150	
BS-1B	2	15	150.1	300						
BS-1AUa	2	16	150.16	300	275	500	125	175	50	150
BS-AUb	2	17	70.04	300	275	625	225	150		
BS-1AUG	2	18	123.15	300	275	575	250	25	150	
BL-1AUa	2	19	70.09	300	275	625	200	25	150	
BL-1AUb	2	20	121.37	300	275	625	225	150		
BL-1AG	2	21	115.96	300	275	625	200	25	150	
CS-1B	2	22	150.84	300						
CS-1AUa	2	23	150.21	300	275	625	175	50	150	

Sample ID	Rep No.	Bottle Label	Soil Sample Mass (g)	Sample Events Effluent Volume (mL)						
				1	2	3	4	5	6	7
CS-1AUb	2	24	85.09	300	275	625	225	150		
CS-1AG	2	25	150.07	300	275	625	225	150		
CL-1AUa	2	26	70.5	300	275	625	200	25	150	
CL-1AUb	2	27	130.13	300	275	575	275	150		
CL-1AG	2	28	123.13	300	275	625	100	75	50	150
BS-1B	3	29	150.23	300						
BS-1AUa	3	30	150.18	300	275	625	175	50	150	
BS-1AUb	3	31	70.03	300	275	625	225	150		
BS-1AUG	3	32	140.58	300	275	625	225	150		
BL-1AUa	3	33	71.03	300	275	625	225	150		
BL-1AUb	3	34	118.15	300	275	625	200	25	150	
BL-1AG	3	35	109.53	300	275	575	225			
CS-1B	3	36	150.53	300						
CS-1AUa	3	37	150.33	300	275	625	175	50	150	
CS-1AUb	3	38	78.31	300	275	625	225	150		
CS-1AG	3	39	150	300	275	625	225	150		
CL-1AUa	3	40	71.91	300	275	625	200	25	150	
CL-1AUb	3	41	139.68	300	275	625	225	150		
CL-1AG	3	42	124.44	300	275	625	225	150		
BS-1B	4	43	136.95	300						
BS-1AUa	4	44	149.86	300	275	625	175	50	150	
BS-1AUG	4	45	133.33	300	275	625	225	150		
BL-1AUb	4	46	121.96	300	275	625	225	150		
BL-1AG	4	47	119.75	300	275	625	225	150		
CS-1B	4	48	151.34	300						
CS-1AUa	4	49	154.16	300	275	450	175	175	50	150
CS-1AG	4	50	153.9	300	275	625	225	150		
CL-1AUb	4	51	130.84	300	275	625	175	50	150	
CL-1AG	4	52	129.56	300	275	625	225	150		

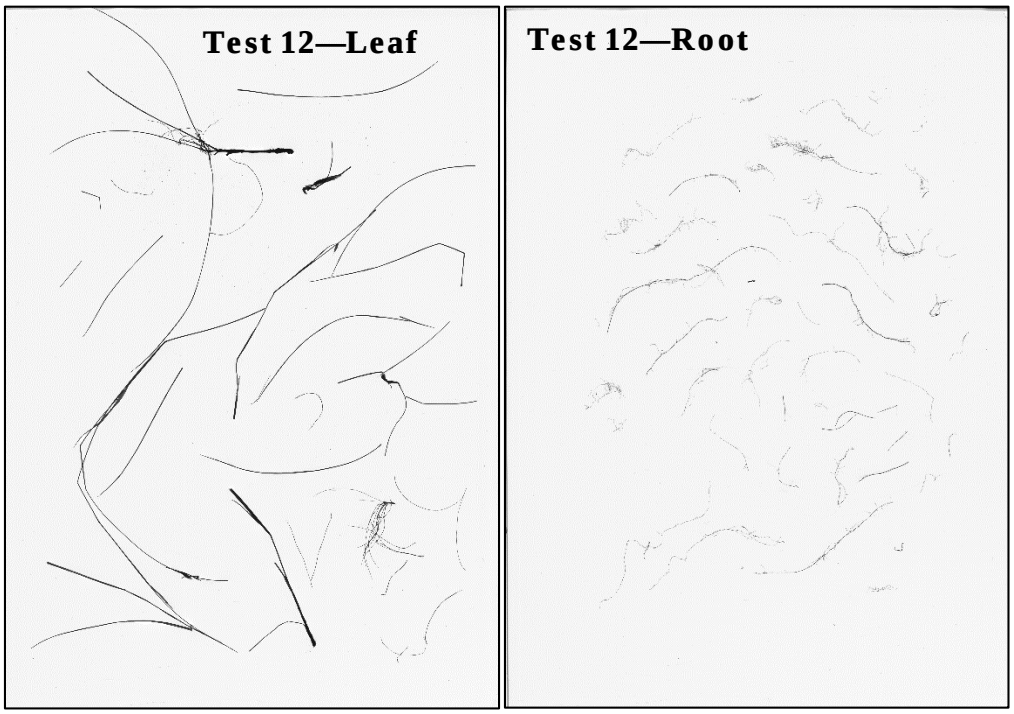
The study used 52 cone sample holders, each with a fiberglass plug placed at the bottom. Between 69 and 155 g of soil was added to each container along with several seeds of the ryegrass (*L. rigidum*). The containers were watered daily with 25 mL of water. There were seven sampling events varying in length from 1 to 25 days, covering the 8 months of the study. The ryegrass germinated in several weeks (Figure 6). In all cases, no vegetative material was recovered for the >2 mm soil material. Sample containers

were placed below the cone holders to capture the effluent (Figure 7), which was analyzed by ICP-OES and ICP-MS. The volume of recovered effluent water varied from 25 to 625 mg (0.25 to 0.625 mL) depending on the soil type, plant uptake, and degree of evapotranspiration. On completion of the experiment, the roots and leaves were recovered and separated. The mass of vegetative material was recorded, and both the roots and leaves were then imaged on a Regent Instruments Inc. LA2400 scanner at a resolution of 800 dpi by using the WinRhizo Pro version 2011b software (Figure 8). Along with providing an image, the software calculates the root or leaf morphology length, surface area, average diameter, volume, number of tips, number of forks, and number of crossings. The scanner is factory calibrated to ensure correct measurements at all resolutions.

Figure 6. Vegetation.

Figure 7. Vegetation uptake experiment holders.

Figure 8. Image of scanned leaf and root sample for Test 12 contaminated loam (CL-1AUa) in <250 µm to >2 mm soil.



3.6 Analytical methods

The ERDC Environmental Laboratory (EL) located in Vicksburg, MS, analyzed the aqueous samples and solid digestates by ICP-OES and ICP-MS following modifications of USEPA methods 6010 (USEPA 2006c) and 6020 (USEPA 2006b), respectively, for the suite of elements reported. Each element was reported from the analytical technique appropriate for the concentrations detected in the matrix. ICP-OES samples were analyzed on a Perkin Elmer Optima 8300DV using a quartz cyclonic spray chamber and MiraMist Nebulizer. Yttrium and Scandium were added in line for use as internal standards to correct for instrumental drift and plasma fluctuations. Samples were analyzed on a Perkin Elmer NexION 300D ICP-MS, which was operated in standard mode and also used a quartz cyclonic spray chamber and MiraMist nebulizer. Scandium, Germanium, Yttrium, Rhodium, Indium, Terbium, Holmium, and Bismuth were added in line for use as internal standards. All calibration and check standards were commercially available from CPI International and SPEX Certiprep and were NIST*-traceable.

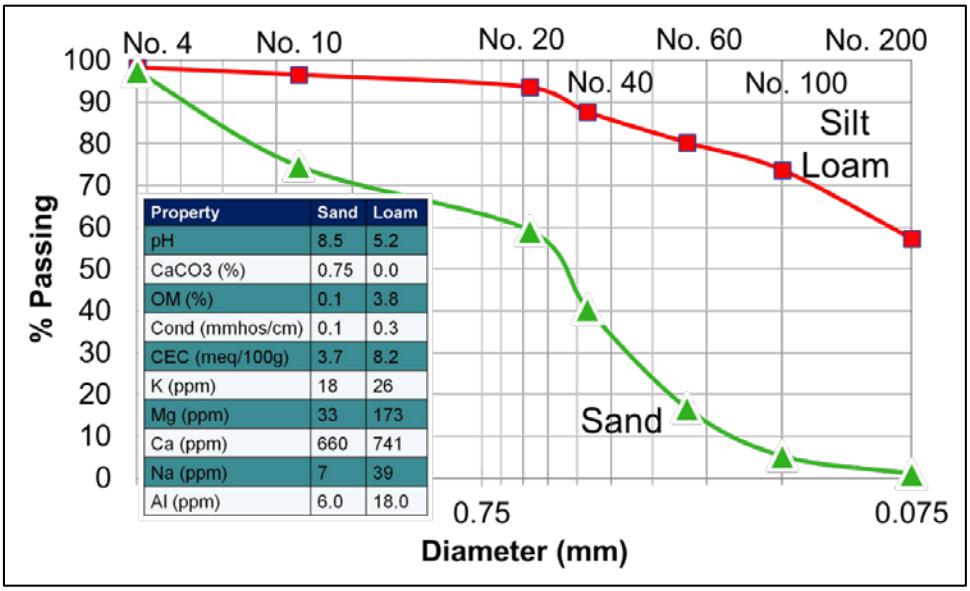
* National Institute of Standards and Tests

4 Results

4.1 Soil properties

Based on particle size analysis, the study material consisted of loam and sand (Figure 9). Two berms containing each material, a study berm, and a control were sampled. The pH was 8.5 for the sand and 5.2 for the loam with the latter have a significantly greater proportion of organic matter. Cation concentrations and cation exchange capacity were higher for the loam versus the sand (Figure 9).

Figure 9. Particle size distribution and general chemical properties for the loam and sand used in this study.



Tables 6 and 7 provide the initial soil concentration based on Method 3050. There was no significant increase in the cations, phosphorous, or silica for the contaminated versus uncontaminated soils (Table 6). Consistent with the projectiles fired into the berm, the contaminated loam and sand had higher levels of antimony, copper, lead, and zinc as compared to the background samples (Table 7). Regarding particle size, the sieved >2 mm material had the lowest concentrations of antimony, copper, lead, and zinc. There was no consistent pattern in metal concentrations between the >250 µm to <2 mm and <250 µm anthropogenic material. The ground material typically had a concentration near the mean of >250 µm to <2 mm and <250 µm material as shown in Figure 10.

Table 6. Initial soil concentration measurements.

Sample ID	Sample Name	Total Dry Mass (kg)	Comment	EL ID	Digestion Date	Analysis Date	Ca	K	Na	Mg	P	Si
							(mg/kg)					
BL-1B	Background Loam	0.61	Sieved >2 mm	4013004-05	29-Jan-14	30-Jan-14	1330	601	24	9570	396	21
BL-1AUa	Background Loam	0.46	Sieved >250 µm to <2 mm	4013004-06	29-Jan-14	30-Jan-14	3160	729	148	4690	453	109
BL-1AUb	Background Loam	3.32	Sieved <250 µm	4013004-07	29-Jan-14	30-Jan-14	2600	656	159	5060	456	150
BL-1AG	Background Loam	7.52	Ground <2 mm	4013004-08	29-Jan-14	30-Jan-14	2850	1200	240	5380	447	234
						Mean	**2485**	**797**	**6175**	**143**	**438**	**129**
CL-1B	Contaminated Loam	1.11	Sieved >2 mm	4013004-13	29-Jan-14	30-Jan-14	966	1150	43.4	1970	248	21.0
CL-1AUa	Contaminated Loam	0.32	Sieved >250 µm to <2 mm	4013004-14	29-Jan-14	30-Jan-14	2450	680	149	3960	353	80.9
CL-1AUb	Contaminated Loam	2.53	Sieved <250 µm	4013004-15	29-Jan-14	30-Jan-14	2360	570	173	4690	413	36.7
CL-1AG	Contaminated Loam	5.60	Ground <2 mm	4013004-16	29-Jan-14	30-Jan-14	2630	1090	253	5020	419	71.5
						Mean	**2102**	**873**	**3910**	**155**	**358**	**53**
BS-1B	Background Sand	6.64	Sieved >2 mm	4013004-01	29-Jan-14	30-Jan-14	2570	433	239	2010	161	47.0
BS-1AUa	Background Sand	3.29	Sieved >250 µm to <2 mm	4013004-02	29-Jan-14	30-Jan-14	5480	764	183	3640	254	57.1
BS-1AUb	Background Sand	0.73	Sieved <250 µm	4013004-03	29-Jan-14	30-Jan-14	6520	758	296	4380	528	62.5
BS-1AG	Background Sand	8.09	Ground <2 mm	4013004-04	29-Jan-14	30-Jan-14	6300	1180	481	3970	286	276
						Mean	**5218**	**784**	**3500**	**300**	**307**	**111**
CS-1B	Contaminated Sand	4.42	Sieved >2 mm	4013004-09	29-Jan-14	30-Jan-14	2420	567	35.3	2330	203	25.8
CS-1AUa	Contaminated Sand	2.68	Sieved >250 µm to <2 mm	4013004-10	29-Jan-14	30-Jan-14	4330	723	217	3190	203	53.1
CS-1AUb	Contaminated Sand	0.64	Sieved <250 µm	4013004-11	29-Jan-14	30-Jan-14	6150	714	285	4100	436	49.7
CS-1AG	Contaminated Sand	6.40	Ground <2 mm	4013004-12	29-Jan-14	30-Jan-14	5340	971	359	3470	257	153
						Mean	**4560**	**744**	**3273**	**224**	**275**	**70**

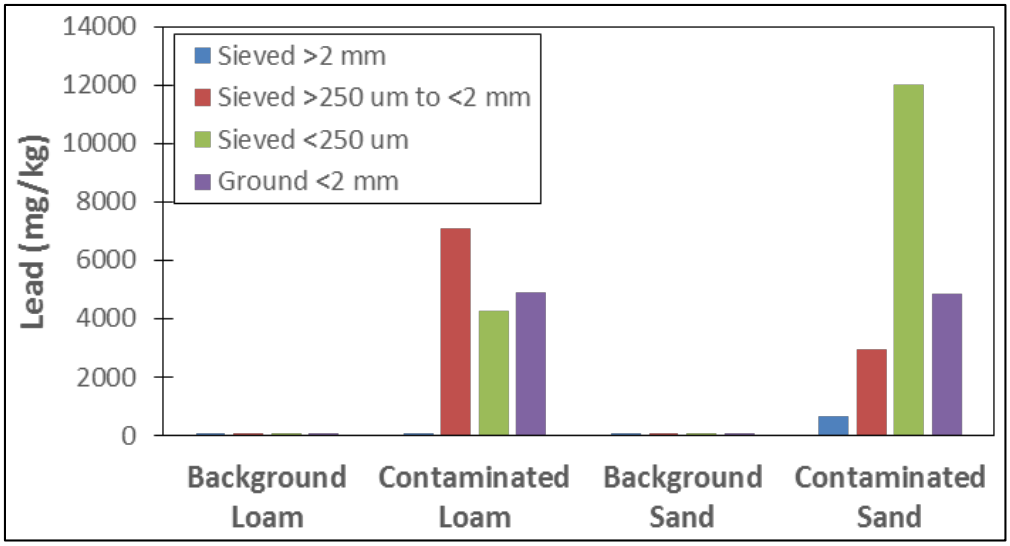
Ca = calcium K = potassium Na = sodium
Mg = magnesium P = phosphorus Si = silicon

Table 7. Initial metal soil concentration (mg/kg) measurements.

Sample Id	Comment	Al	Sb	As	Ba	Cr	Co	Cu	Fe	Pb	Mn	Ni	V	Zn
		(mg/kg)												
BL-1B	Sieved >2 mm	12400	<1.00	6.62	51.6	24.0	15.3	31.6	28700	4.08	384	27.1	74.7	30.9
BL-1AUa	Sieved >250 µm to <2 mm	12600	<1.00	25.7	121	20.9	13.9	33.0	30200	15.4	405	27.5	38.2	37.1
BL-1AUb	Sieved <250 µm	13400	<1.00	15.4	108	21.2	10.5	24.5	25800	11.1	252	24.1	32.7	39.7
BL-1AG	Ground <2 mm	14600	<1.00	15.7	125	132	11.1	26.0	27700	11.5	284	25.9	35.1	40.3
	Mean	**13250**	**<1.00**	**16**	**101**	**50**	**13**	**29**	**28100**	**11**	**331**	**26**	**45**	**37**
CL-1B	Sieved >2 mm	3440	<1.00	2.37	27.5	3.83	3.66	5.91	8090	50.9	157	4.65	13.2	16.0
CL-1AUa	Sieved >250 µm to <2 mm	10400	29.3	28.5	101	<1.00	12.0	<1.00	32100	7080	412	27.2	33.6	4360
CL-1AUb	Sieved <250 µm	12600	13.8	17.8	101	16.5	10.1	517	24700	4250	246	23.0	31.4	90.8
CL-1AG	Ground <2 mm	14000	17.3	19.1	120	85.6	11.1	4270	28000	4900	292	25.7	34.0	510
	Mean	**10110**	**20**	**17**	**87**	**35**	**9**	**1598**	**23223**	**4070**	**277**	**20**	**28**	**1244**
BS-1B	Sieved >2 mm	3430	<1.00	6.45	19.8	6.03	3.64	16.8	6870	2.21	149	9.79	12.9	13.1
BS-1AUa	Sieved >250 µm to <2 mm	5470	<1.00	2.63	49.3	9.20	4.36	12.3	9670	3.65	194	11.7	16.6	20.3
BS-1AUb	Sieved <250 µm	6820	<1.00	9.59	55.7	10.9	6.71	18.8	15800	15.6	289	17.0	19.5	29.8
BS-1AG	Ground <2 mm	7750	<1.00	3.65	70.9	220	5.50	13.7	13000	3.55	249	14.6	21.0	22.7
	Mean	**5868**	**<1.00**	**6**	**49**	**62**	**5**	**15**	**11335**	**6**	**220**	**13**	**18**	**21**
CS-1B	Sieved >2 mm	3070	5.18	1.91	52.4	4.02	4.38	50.0	9140	644	298	7.61	15.2	23.3
CS-1AUa	Sieved >250 µm to <2 mm	5280	16.6	4.91	36.8	<1.00	3.90	4100	8970	2930	183	10.5	14.4	486
CS-1AUb	Sieved <250 µm	6030	141	24.2	46.3	<1.00	5.78	2290	14300	12000	272	15.7	16.1	282
CS-1AG	Ground <2 mm	6380	14.0	7.46	59.5	107	4.68	1550	11500	4820	221	13.6	17.6	195
	Mean	**5190**	**44**	**10**	**49**	**56**	**5**	**1998**	**10978**	**5099**	**244**	**12**	**16**	**247**

Al = aluminum Sb = antimony As = arsenic Ba = barium Cr = chromium
Co = cobalt Cu = copper Fe = iron Pb = lead Mn = manganese
Ni = nickel V = vanadium Zn = Zinc

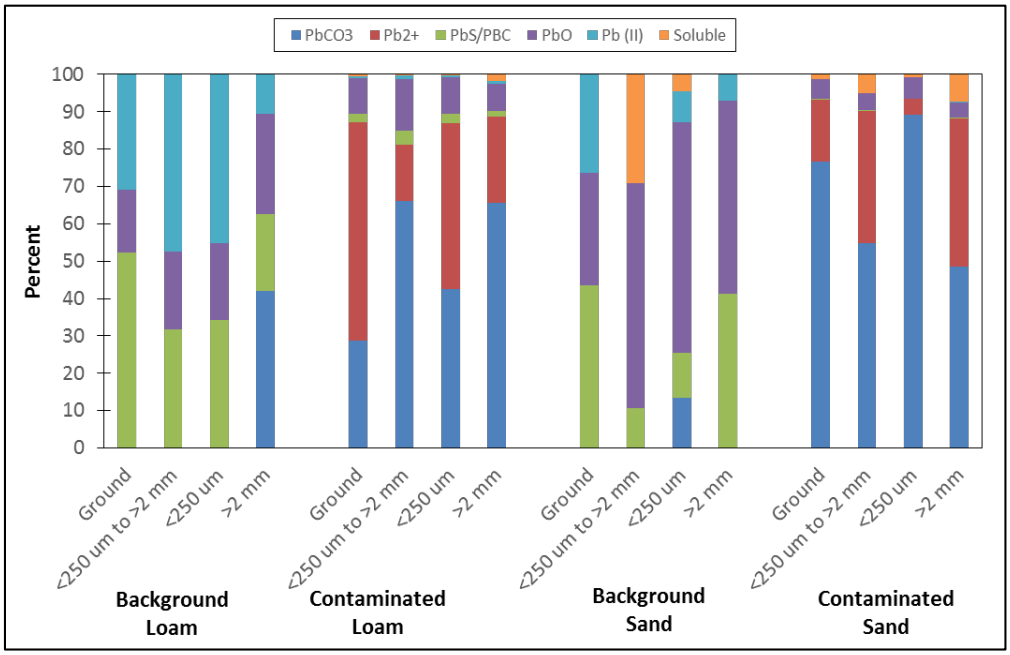
Figure 10. Lead soil concentrations for background and contaminated study materials.



4.1.1 Lead speciation

The speciation of lead was determined using the methods of Baumann and Fisher (2011) and Tessier et al. (1979). The background loam samples primarily consist of lead sulfide (PbS) or organic lead (PbC) species, exchangeable lead (Pb(II)), and lead oxide (PbO) (Figure 11). The background sand was similar to the background loam but with more PbO and some soluble lead (PbSol).

Figure 11. Lead speciation for study soils.

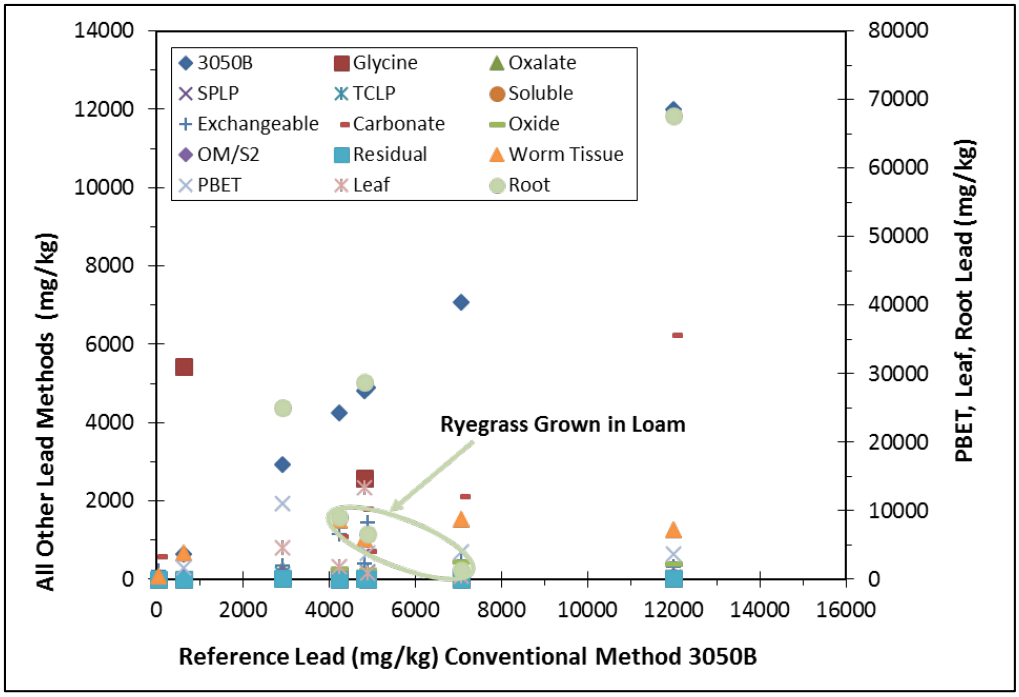


In contrast, the predominant lead species were different for the contaminated material with a preponderance of lead carbonate ($PbCO_3$) and residual lead (Pb^{2+}). There was an absence of PbS/PbC and a presence of PbSol in the contaminated sand as compared to the contaminated loam. There was only a slight difference in the lead distribution pattern between the different particle sizes or as compared with the milled material.

4.1.2 Other digestion approaches

Other digestion approaches evaluated and compared against the sequential digestion procedure above and Method 3050 included the use of glycine, EDTA, oxalate, SPLP, and TCLP. In comparison with Method 3050, most of the alternative digestion tests yielded lower recoveries of lead (Figure 12). The best recovery of lead with results comparable to the soil lead recoveries with Method 3050 were the rye grass root samples grown in sand. In contrast, the rye grass root samples grown in loam had much lower recoverable lead (Figure 12).

Figure 12. Various lead soil concentrations by digestion method compared with Method 3050B.



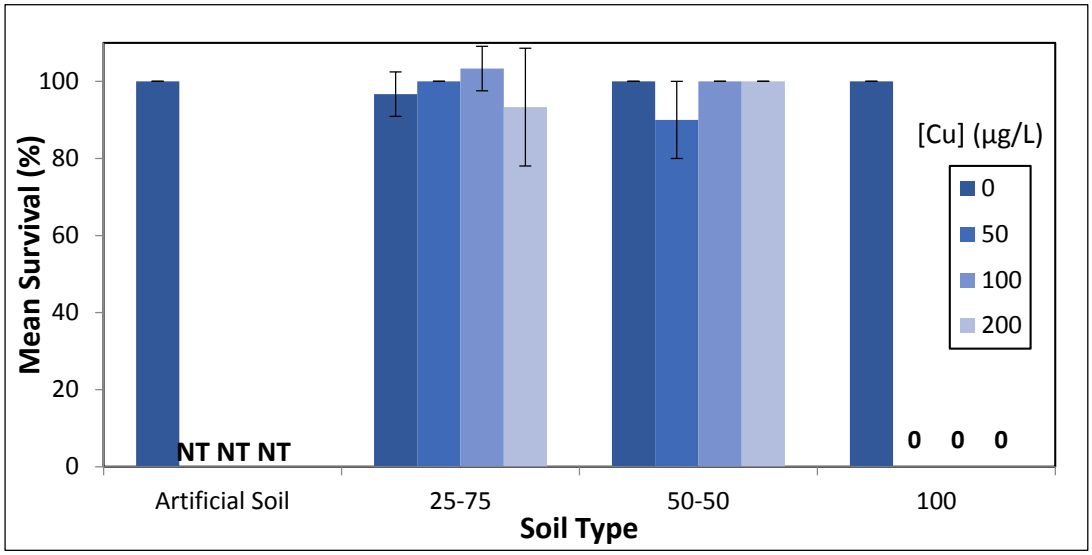
4.2 Earthworm bioaccumulation experiments

4.2.1 Phase I—Particle size impacts

Survival data for Phase I toxicity tests with the earthworm *E. fetida* are summarized in Figure 13. The control soil passed test acceptability criteria with 100% survival. For all soil mixtures, high survival was observed in unspiked treatments and ranged from 97% to 100%. For the soil mixture that was 25% sand and 75% artificial soil, survival was high across all copper concentrations, ranging from 93% to 100%. For the 50:50 sand:artificial soil mixture, survival was also high across all concentrations (mean survival ranged 90% to 100%). In the 100% sand treatment, only the unspiked sample resulted in high survival (100%). All spiked concentrations of the 100% sand treatment resulted in complete mortality.

The high mortality rate in the 100% sand treatment may be related to increased bioavailability of copper versus that in the artificial soil. The artificial soil used as a control and as a diluting material had a 10% peat moss content, which possibly acted as a sink to the copper and rendered the copper less bioavailable to the earthworms. Additionally, the 100% sand matrix was different, from a grain size and physical standpoint, than the earthworms' normal habitat, which is closer the artificial soil matrix. These results prompted the use of 50:50 artificial soil:ISM test soil for subsequent tests.

Figure 13. Mean percent earthworm survival (±SD) from spiking studies.



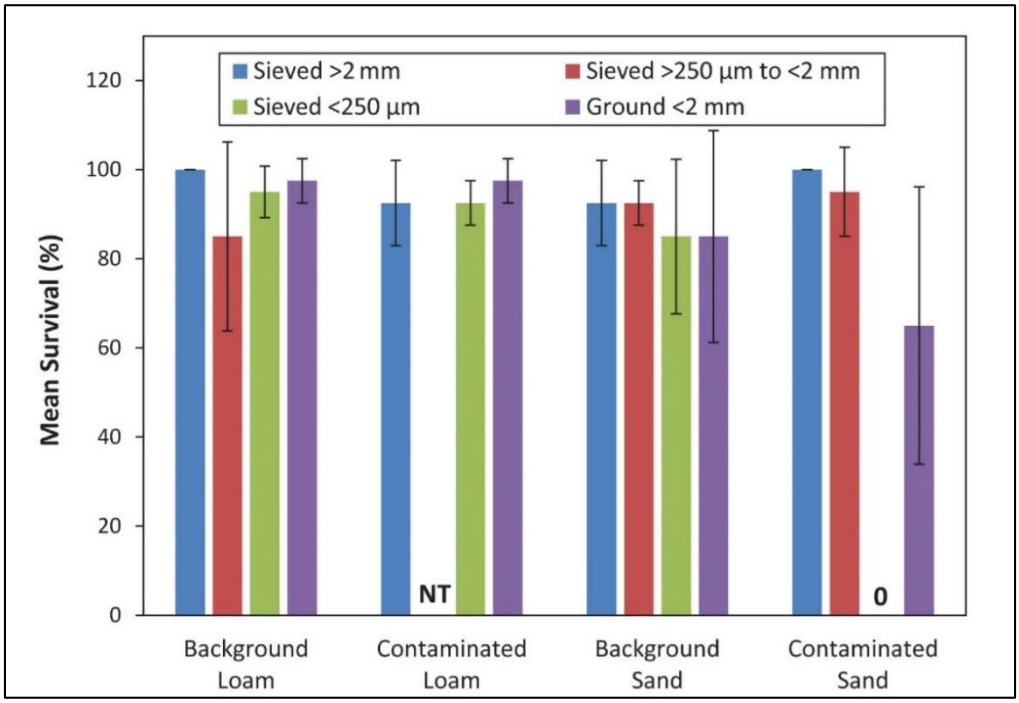
0 = zero percent survival, NT = not tested, SD = standard deviation

4.2.2 Phase II—Soil toxicity

The earthworm survival, growth, and bioaccumulation tests met all applicable control and testing condition quality criteria. All worms recovered at the end of the exposure period appeared healthy based on visual color and activity. Activity included burrowing ability and reaction to stimuli.

Mean survival among all soil types and the artificial soil control at day 14 ranged from 0% to 100% (Figure 14). Each soil type was compared statistically against the artificial soil control by using two-sample t-tests. Only the contaminated sand sample that was sieved to <250 µm was statistically different from the control with complete mortality in this treatment. Because of the observed mortality, it was of concern that if the contaminated sand samples were left to continue for the remainder of the test period, high levels of mortality may occur, resulting in a lack of usable data at that time. Thus, the decision was made to terminate all the sand experiments at 14 days. For comparative purposes, a single worm was removed from each loam sample replicate and depurated for 24 hr and then preserved alongside the sand samples until tissue digestion and subsequent ICP analysis.

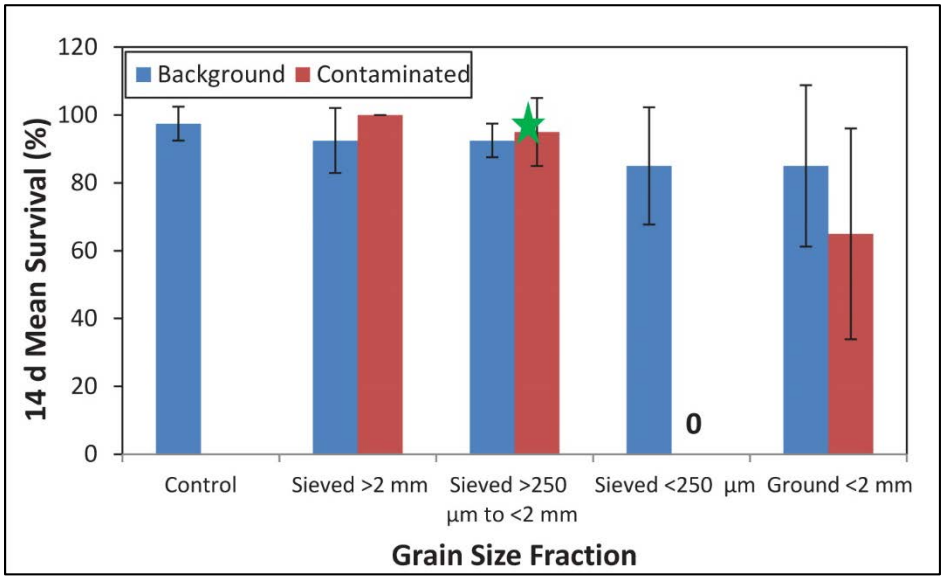
Figure 14. Earthworm 14-day mean survival (±SD) in all samples.



0 = zero percent survival, NT = not tested, SD = standard deviation

Further evaluation of the 14-day sand samples included statistical comparison of the background to the contaminated samples within each grain size fraction. Mean survival ranged from 85% to 92% and 0% to 100% for the background and contaminated samples, respectively (Figure 15; Table 8). There were no statistical differences for the 14-day survival data, except for the <250 µm sieved size fraction, in which the contaminated sample had 0% survival and the background sample had 85.0 ± 17.3% survival (two-sample t-test: $p = 0.002$). The mean survival between background and contaminated size fractions differed by approximately 20% but was not statistically different (two-sample t-test: $p = 0.104$).

Figure 15. Earthworm 14-day mean survival (±SD) in sand.



The green star indicates statistically significant difference between background and contaminated samples within that size fraction.

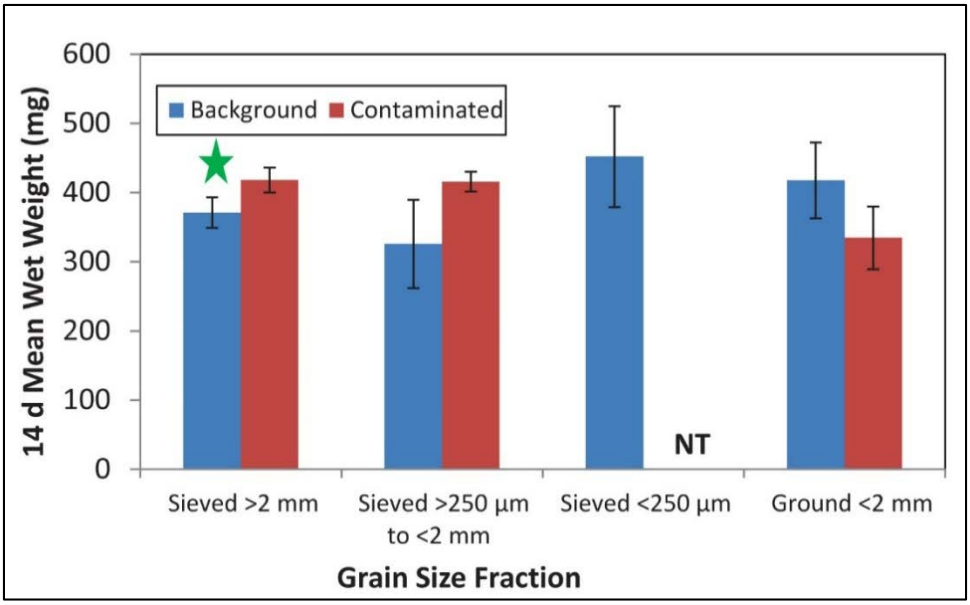
0 = zero percent survival, SD = standard deviation

Table 8. Earthworm 14-day survival in sand.

Grain Size Fraction/Treatment	Earthworm Mean 14-Day Survival (%)	
	Background	Contaminated
Control	97.5 ± 5.0	-
Sieved >2 mm	92.5 ± 9.6	100 ± 0.0
Sieved >250 µm to <2 mm	92.5 ± 5.0	95.0 ± 10.0
Sieved <250 µm	85.0 ± 17.3	0.0 ± 0.0
Ground <2 mm	85.0 ± 23.8	65.0 ± 31.1

Mean individual wet weights of the worms following the 14-day test period ranged from 326 to 452 mg and 334 to 418 mg for the background and contaminated samples, respectively (Figure 16; Table 9). Significant differences were observed between the background and contaminated samples for the >2 mm size fractions (two-sample t-test: $p = 0.017$).

Figure 16. Earthworm 14-day mean wet weight (±SD) in sand.



The green star indicates statistically significant difference between background and contaminated samples within that size fraction.

NT = not tested, SD = standard deviation

Table 9. Earthworm 14-day mean Individual wet weight (± SD) in sand.

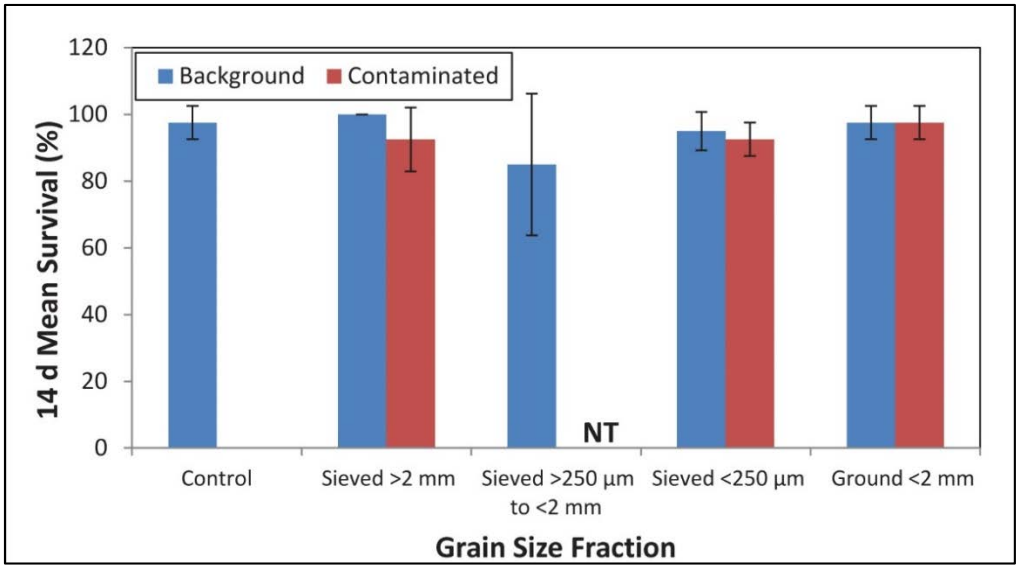
Grain Size Fraction/Treatment	Earthworm Mean 28-day Individual Wet Weight (mg)	
	Background	Contaminated
Sieved >2 mm	371 ± 22.0	418 ± 17.8
Sieved >250 µm to <2 mm	326 ± 63.7	416 ± 14.2
Sieved <250 µm	452 ± 73.0	NT
Ground <2 mm	417 ± 54.7	334 ± 45.2

NT = not tested

Survival was the only variable evaluated at 14-days for the loam samples as the worms were allowed to continue for the full 28-day exposure period (Figure 13 and Figure 17; Table 10). Mean control survival was 97%. Mean survival ranged from 85% to 100% and 92% to 97% for the background and contaminated samples, respectively. There were no significant differ-

ences between any of the loam samples and the artificial control. Comparing the background and contaminated loam samples, there was no significant difference looking at each grain size fraction individually ($p > 0.05$); no comparison was made for the <2 mm fraction as there was no contaminated <2 mm sample tested (Figure 17).

Figure 17. Earthworm 14-day mean survival (±SD) in loam.



NT = not tested, SD = standard deviation

Table 10. Earthworm 14-day survival in loam.

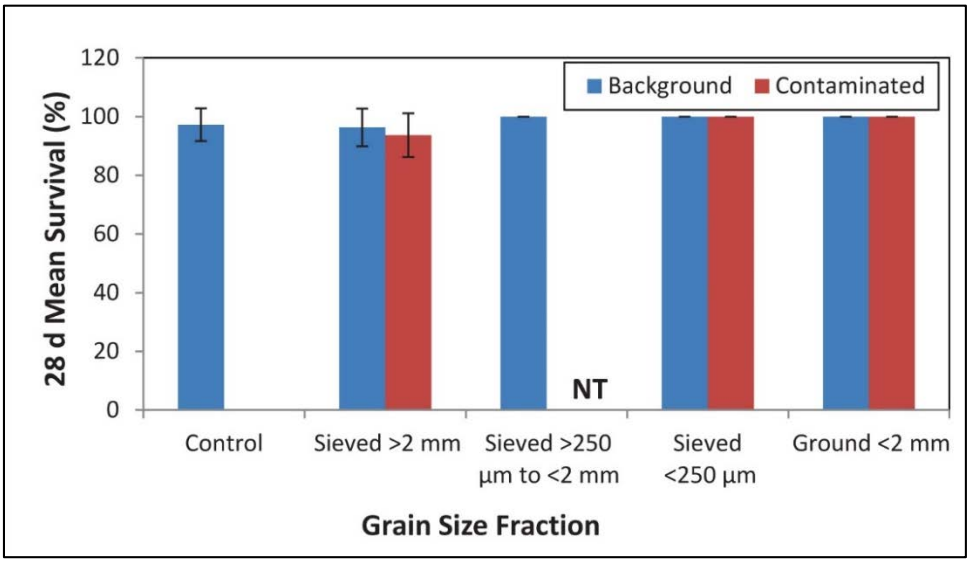
Grain Size Fraction/Treatment	Earthworm Mean 14-Day Survival (%)	
	Background	Contaminated
Control	97.5 ± 5.0	-
Sieved >2 mm	100 ± 0.0	92.5 ± 9.6
Sieved >250 µm to <2 mm	85.0 ± 21.2	NT
Sieved <250 µm	95.0 ± 5.8	92.5 ± 5.0
Ground <2 mm	97.5 ± 5.0	97.5 ± 5.0

NT = not tested

Because a single worm was removed from each replicate of the loam samples at day 14, 28-day survival was based off of the remaining survivors at the 14-day mark (Figure 18; Table 11). Mean control survival was 97%. Mean survival ranged from 96% to 100% and 93% to 100% for the background and contaminated loam samples, respectively. There were no sig-

nificant differences between the control samples and any of the loam samples tests. Comparing background and contaminated samples for individual grain size fractions, there were no significant differences ($p > 0.05$).

Figure 18. Earthworm 28-day mean survival (±SD) in loam.



NT = not tested, SD = standard deviation.

Table 11. Earthworm 28-day survival in loam.

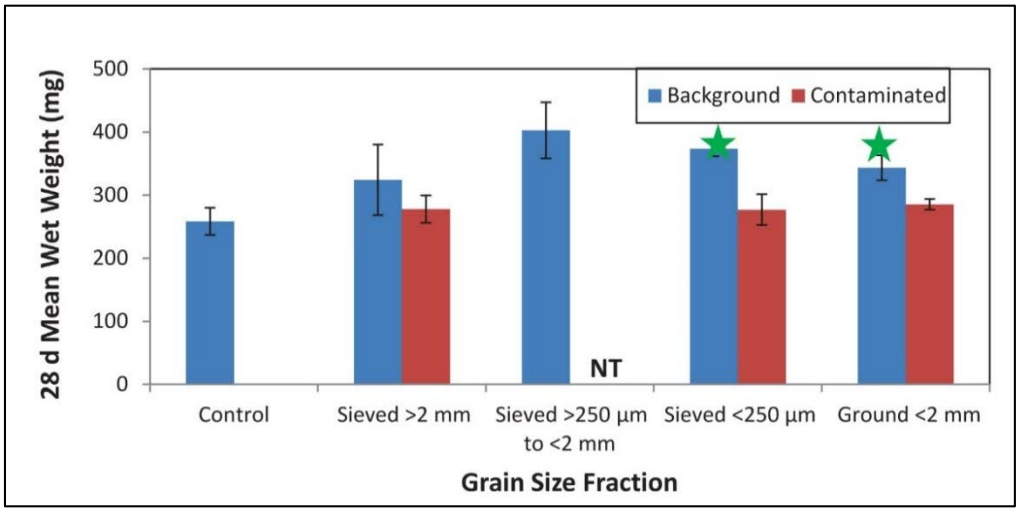
Grain Size Fraction/Treatment	Earthworm Mean 14-Day Survival (%)	
	Background	Contaminated
Control	97.2 ± 5.6	-
Sieved >2 mm	96.3 ± 6.4	93.7 ± 7.4
Sieved >250 µm to <2 mm	100 ± 0.0	NT
Sieved <250 µm	100 ± 0.0	100 ± 0.0
Ground <2 mm	100 ± 0.0	100 ± 0.0

NT = not tested

Control mean individual wet weight at test termination was 258 mg (Figure 19; Table 12). Mean individual wet weights of the worms ranged from 324 to 402 mg and 277 to 285 mg for the background and contaminated samples, respectively. Compared with the artificial soil control, there were significant differences for the background <250 µm and ground samples (two-sample t-tests; <250 µm: $p = 0.0004$, ground: $p = 0.001$), which had greater mean wet weight relative to the control. There were no significant differences in the contaminated samples compared against the control.

There were also significant differences between the background and contaminated samples for the <250 µm and the ground size fraction samples (two-sample t-test; <250 µm: $p = 0.002$; ground: $p = 0.005$).

Figure 19. Earthworm 28-day mean wet weight (±SD) in loam.



The green stars indicate significant difference between background and contaminated samples within that size fraction.
NT = not tested, SD = standard deviation

Table 12. Earthworm 28-day mean individual wet weight (±SD) in loam.

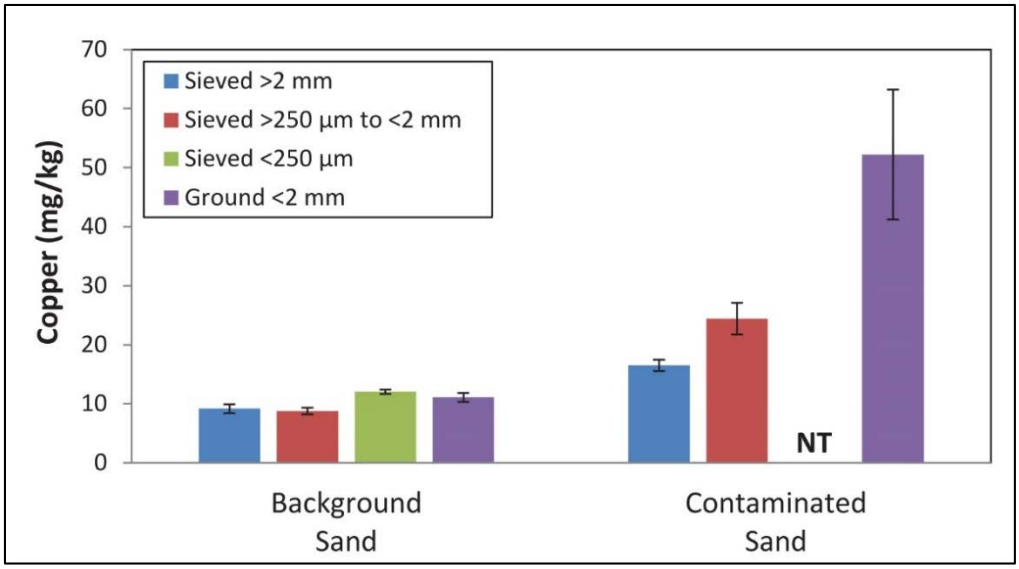
Grain Size Fraction/Treatment	Earthworm Mean 28-Day Individual Wet Weight (mg)	
	Background	Contaminated
Control	258 ± 21.6	-
Sieved >2 mm	324 ± 55.8	278 ± 21.7
Sieved >250 µm to <2 mm	402 ± 44.5	NT
Sieved <250 µm	373 ± 11.7	277 ± 24.4
Ground <2 mm	343 ± 19.6	285 ± 8.4

NT = not tested, SD = standard deviation

4.2.3 Worm tissue metals bioaccumulation

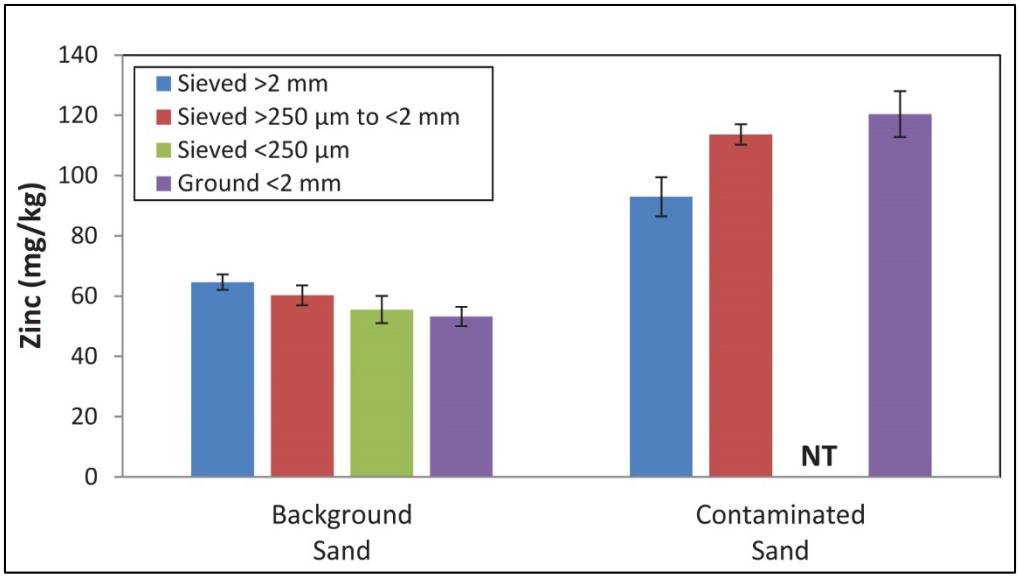
The amount of tissue recovered at test termination was adequate for the required chemical analyses for all soil samples. For both background and contaminated sample types, copper, zinc, lead, and antimony were detected in all grain size fractions for both 14- and 28-day studies (Figures 20–27; Tables 13–14).

Figure 20. Earthworm 14-day copper bioaccumulation (mg/kg) in sand.



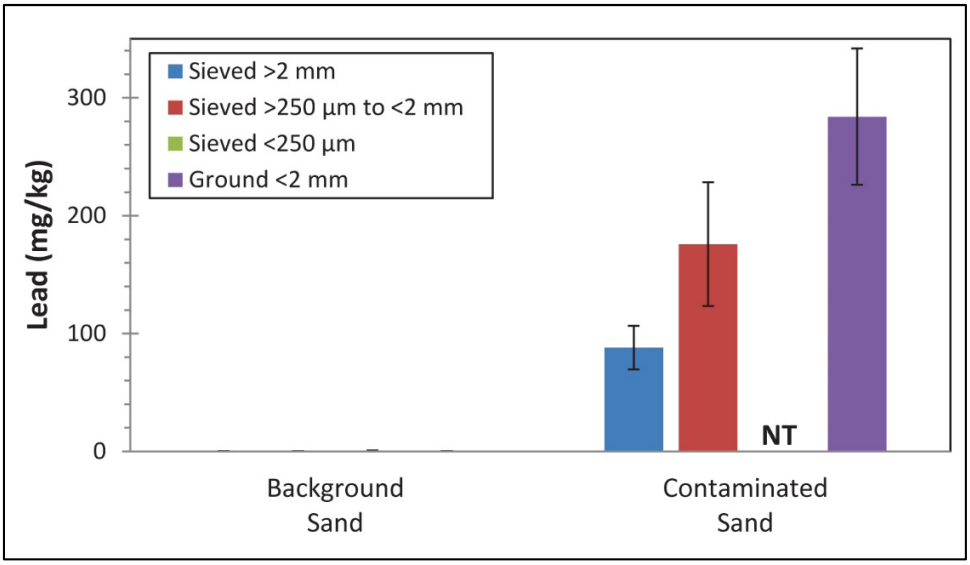
NT = not tested

Figure 21. Earthworm 14-day zinc bioaccumulation (mg/kg) in sand.



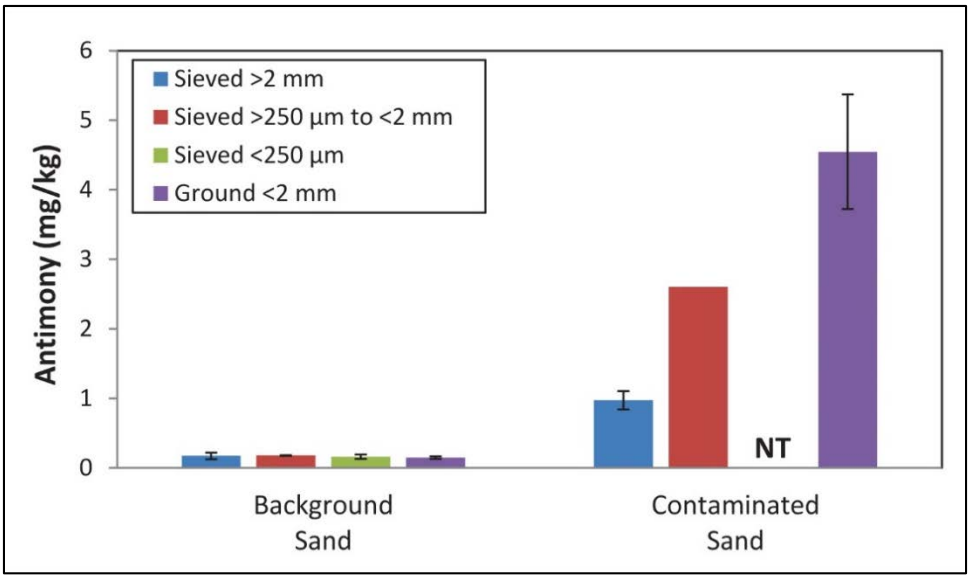
NT = not tested

Figure 22. Earthworm 14-day lead bioaccumulation (mg/kg) in sand.



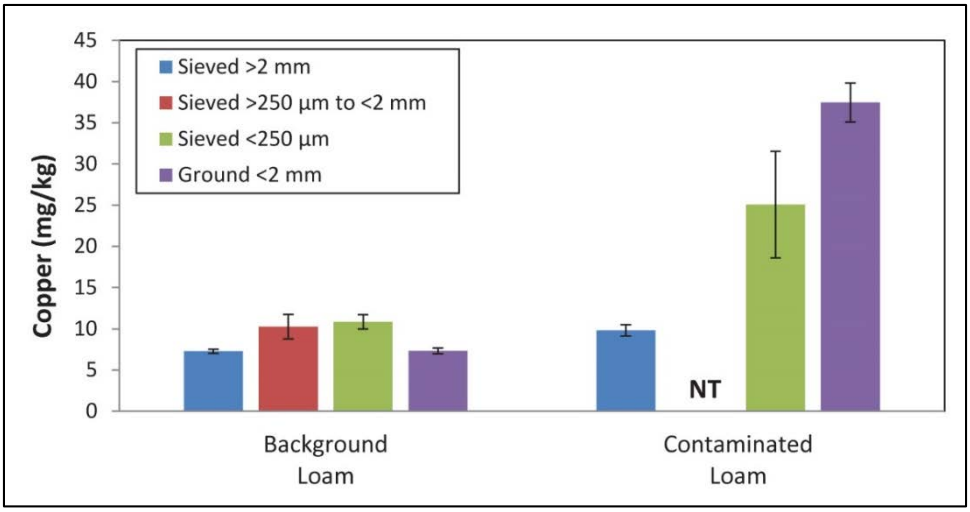
NT = not tested

Figure 23. Earthworm 14-day antimony bioaccumulation (mg/kg) in sand.



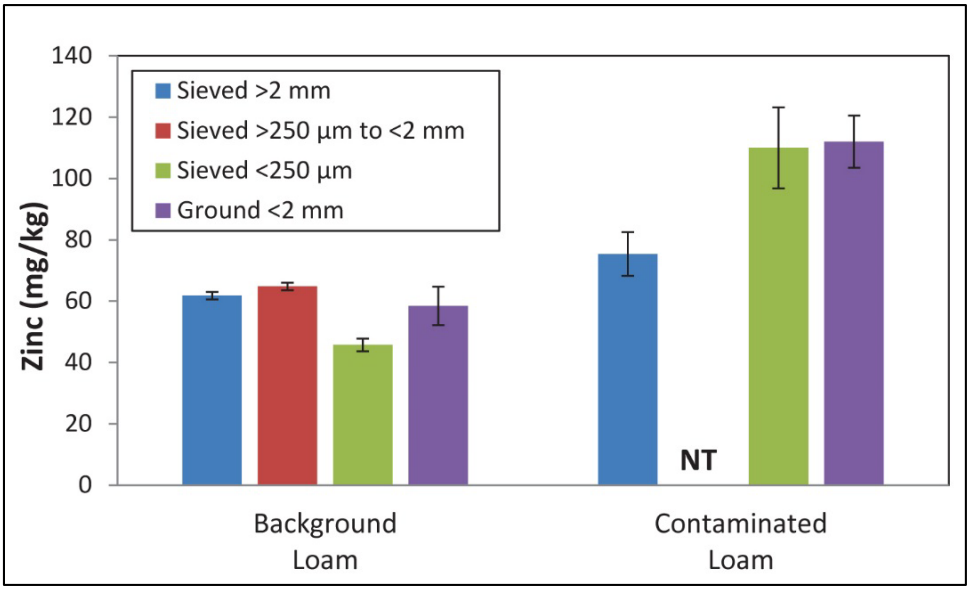
NT = not tested

Figure 24. Earthworm 28-day copper bioaccumulation (mg/kg) in loam.



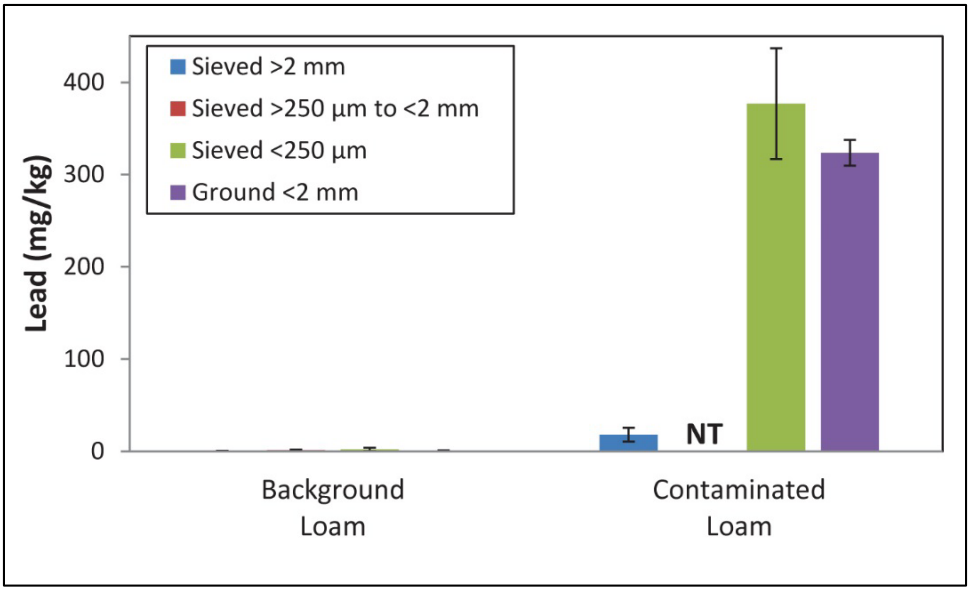
NT = not tested

Figure 25. Earthworm 28-day zinc bioaccumulation (mg/kg) in loam.



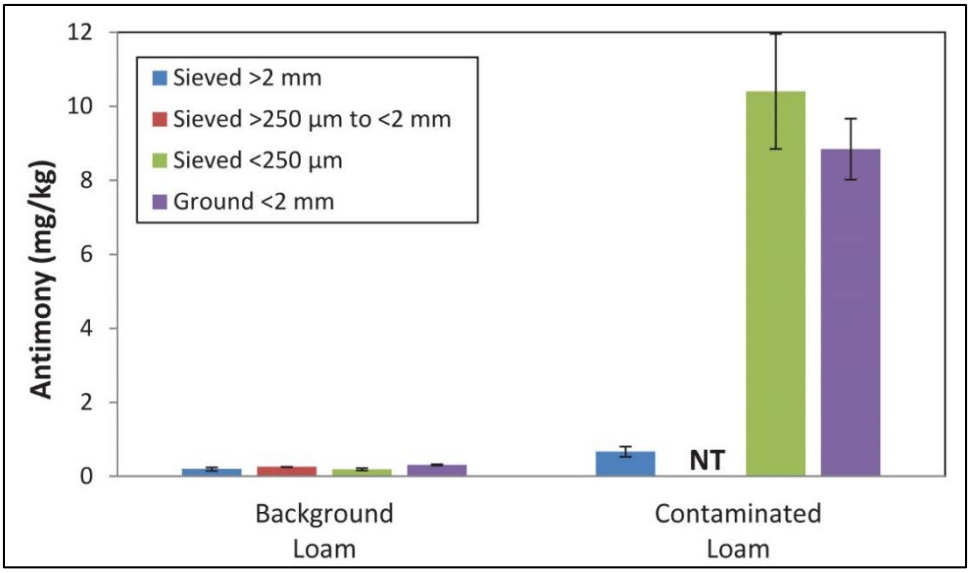
NT = not tested

Figure 26. Earthworm 28-day lead bioaccumulation (mg/kg) in loam.



NT = not tested

Figure 27. Earthworm 28-day antimony bioaccumulation (mg/kg) in loam.



NT = not tested

Table 13. Earthworm 14-day tissue metal concentrations (mg/kg) wet weight (±SD) in sand.

Grain Size Fraction/ Treatment	Earthworm 14-Day Tissue Metal Concentrations (mg/kg)							
	Background				Contaminated			
	Cu	Zn	Pb	Sb	Cu	Zn	Pb	Sb
Sieved >2 mm	9.2	64.7	0.3	0.2	16.5	93.0	88.2	1.0
Sieved >250 µm to <2 mm	8.8	60.3	0.3	0.2	24.4	114	176	2.6
Sieved <250 µm	12.1	55.5	0.7	0.2	NT	NT	NT	NT
Ground <2 mm	11.1	53.3	0.2	0.1	52.2	120	284	4.5

NT = not tested, SD = standard deviation

Table 14. Earthworm 28-day tissue metal concentrations (mg/kg) wet weight (±SD) in loam.

Grain Size Fraction/Treatment	Earthworm 28-day Tissue Metal Concentrations (mg/kg)							
	Background				Contaminated			
	Cu	Zn	Pb	Sb	Cu	Zn	Pb	Sb
Sieved >2 mm	7.3	61.8	0.6	0.2	9.8	75.4	18.0	0.7
Sieved >250 µm to <2 mm	10.3	64.8	1.3	0.3	NT	NT	NT	NT
Sieved <250 µm	10.9	45.8	2.1	0.2	25.1	110	377	10.4
Ground <2 mm	7.3	58.5	0.9	0.3	37.5	112	324	8.8

NT = not tested, SD = standard deviation

4.2.4 Soil metal concentrations

Presented below in Tables 15 and 16 are the metal concentrations associated with the bulk soil samples that were mixed on a 50:50 basis with artificial soil for the earthworm bioassay exposure. There were positive correlations for all metals evaluated between soil and tissue (Figures 28–31; r^2: Cu = 0.68, Zn = 0.77, Pb = 0.98, Sb = 0.91).

Table 15. Summary of metal concentrations (mg/kg) in sand.

Grain Size Fraction/Treatment	Sediment Metal Concentrations (mg/kg)							
	Background				Contaminated			
	Cu	Zn	Pb	Sb	Cu	Zn	Pb	Sb
Sieved >2 mm	14	81	4	2	45	85	199	5
Sieved >250 µm to <2 mm	12	63	6	0.2	1890	334	1030	27
Sieved <250 µm	11	61	11	1	749	204	5965	149
Ground <2 mm	13	68	4	0.4	964	150	2540	54

Table 16. Summary of metal concentrations (mg/kg) in loam.

Grain Size Fraction/Treatment	Sediment Metal Concentrations (mg/kg)							
	Background				Contaminated			
	Cu	Zn	Pb	Sb	Cu	Zn	Pb	Sb
Sieved >2 mm	14	81	4	2	142	107	355	6.5
Sieved >250 µm to <2 mm	22	108	9	1	NT	NT	NT	NT
Sieved <250 µm	18	92	13	0.7	284	165	2736	49
Ground <2 mm	18	72	8	2	836	234	2710	47

NT = not tested

Figure 28. Soil to earthworm-tissue concentration comparisons for copper.

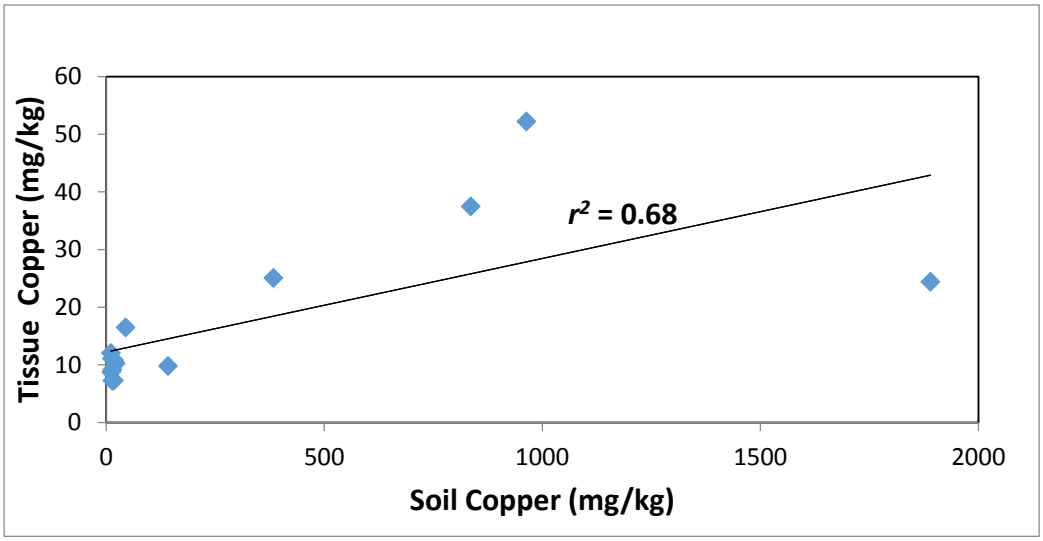


Figure 29. Soil to earthworm-tissue concentration comparisons for zinc.

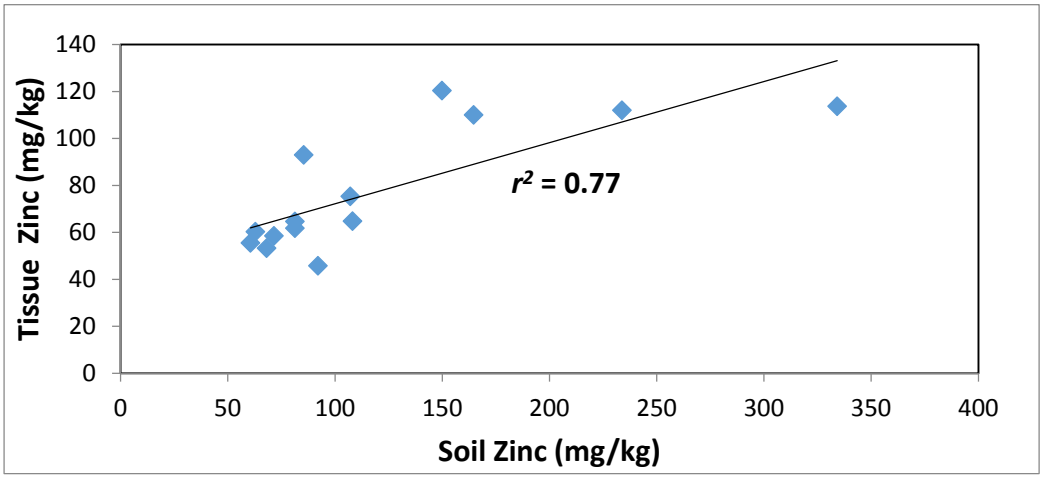


Figure 30. Soil to earthworm-tissue concentration comparisons for lead.

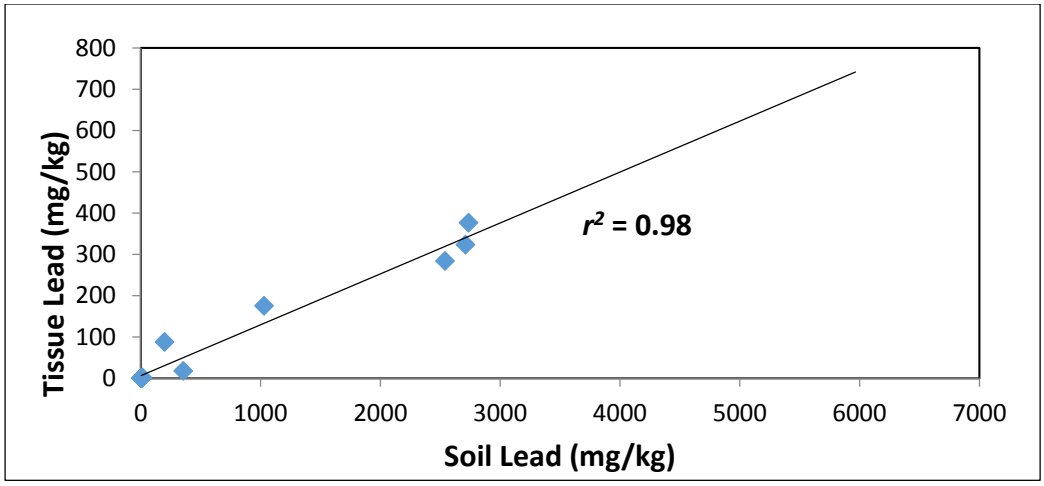
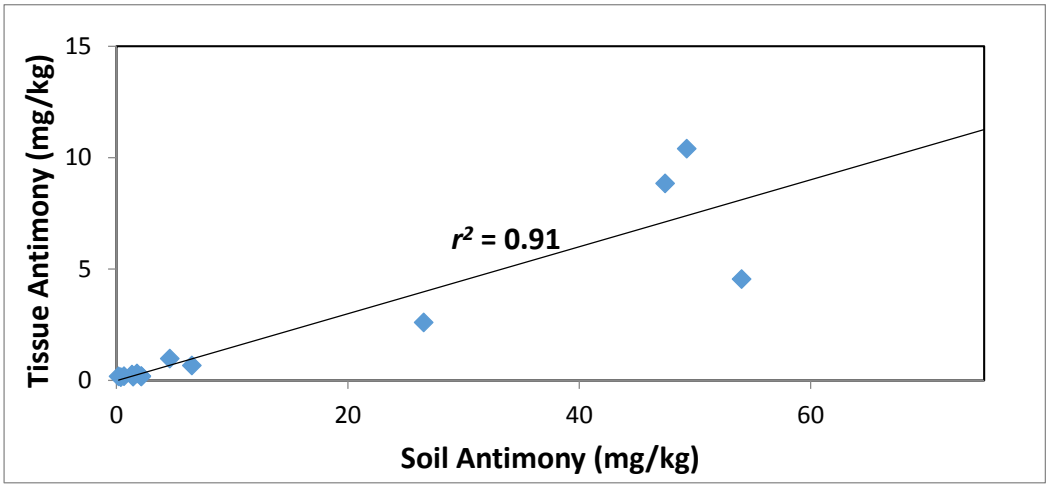


Figure 31. Soil to earthworm-tissue concentration comparisons for antimony.



4.2.5 Diffusive gradients in thin films (DGT) bioavailability assessment

DGT concentrations are a direct measure of the mean flux of labile species originating from the soil sample and can be interpreted directly as the mean concentration of labile metal at the interface between the device surface and the soil during the deployment. Figures 32–35 show the DGT flux from the soil samples. DGT concentrations had positive correlations with bulk soil concentrations for Cu, Zn, and Pb (r^2: Cu = 0.13, Zn = 0.18, Pb = 0.71). The highest flux was in the contaminated sand <250 µm (unmilled) sample. DGT concentrations had positive correlations with worm-tissue concentrations for all metals evaluated (r^2: Cu = 0.98, Zn = 0.84, Pb = 0.16, Sb = 0.38). Note that because of the large particle sizes associated

with the >2 mm size fraction, the DGT protocol was deemed inappropriate and was not performed.

Figure 32. Diffusive gradients in thin films for copper flux.

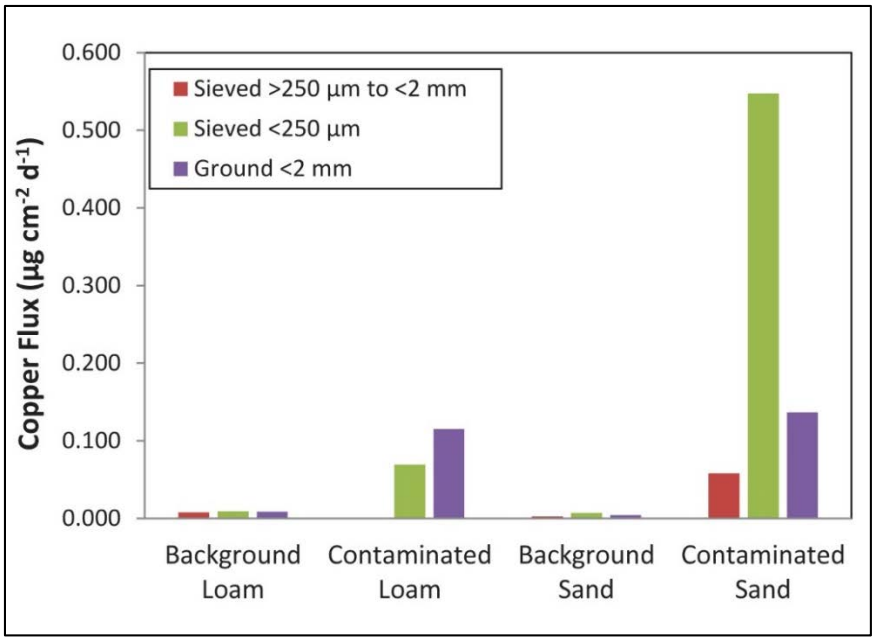


Figure 33. Diffusive gradients in thin films for zinc flux.

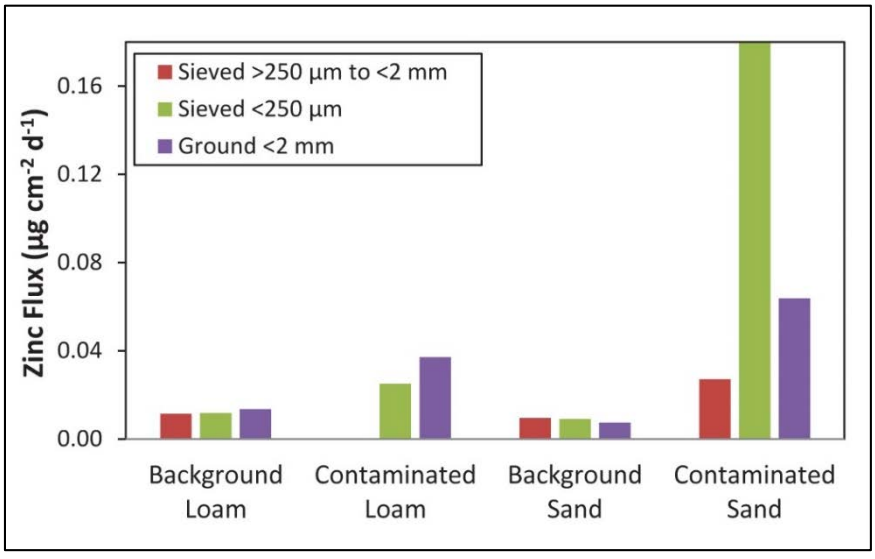


Figure 34. Diffusive gradients in thin films for lead flux.

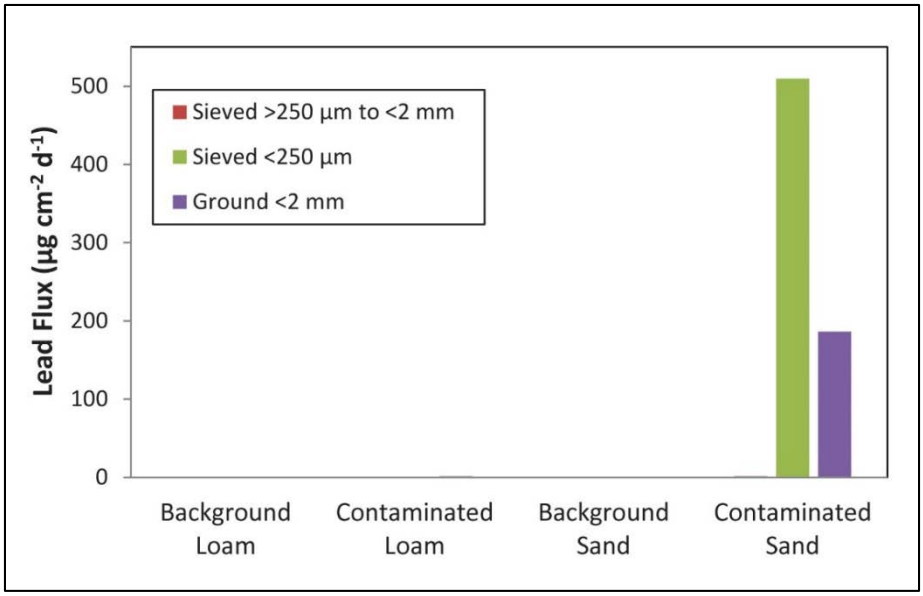
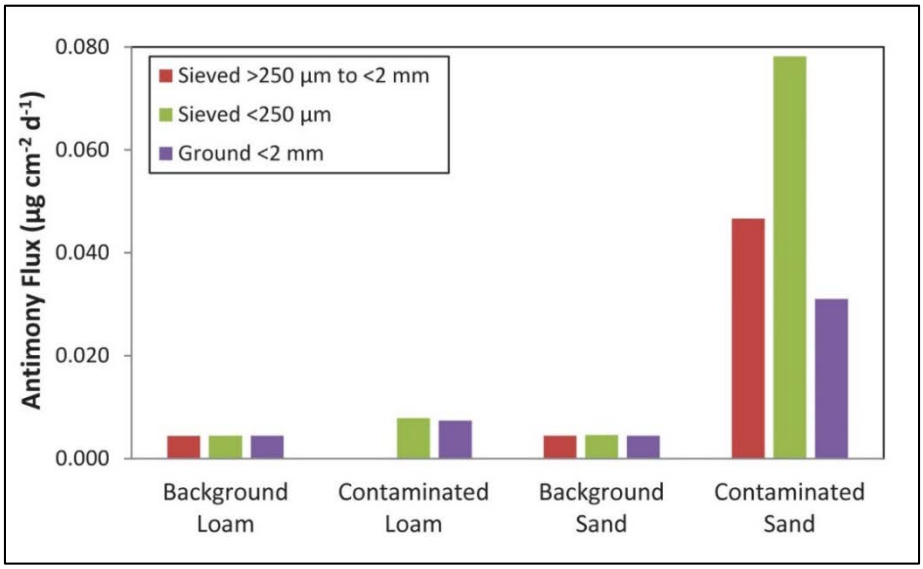


Figure 35. Diffusive gradients in thin films for antimony flux.



4.2.6 Physiologically based extraction technique (PBET) metal bioaccessibility

PBET concentrations had positive correlations with bulk soil concentrations for all soils evaluated (r^2: Cu = 0.50, Zn = 0.53, Pb = 0.99, Sb = 0.99). PBET concentrations also had positive correlations with worm-tissue concentrations (r^2: Cu = 0.94, Zn = 0.79, Pb = 0.81; Sb = 0.98). PBET was a better predictor for metal bioavailability over DGT methods for Pb

and Sb whereas DGT was a more accurate predictor of metal bioavailability for Cu and Zn. Figures 36–39 show PBET results in the form of metal bioaccessibility, which is calculated by dividing the PBET mean concentration by the concentration found in the bulk soil sample. Metal bioaccessibility was higher for the lead soils, indicating greater uptake potential versus Cu or Zn. Metal bioaccessibility was also generally greater in the <250 µm (unmilled) and the <2 mm ground samples.

Figure 36. Physiologically based extraction technique copper bioaccessibility.

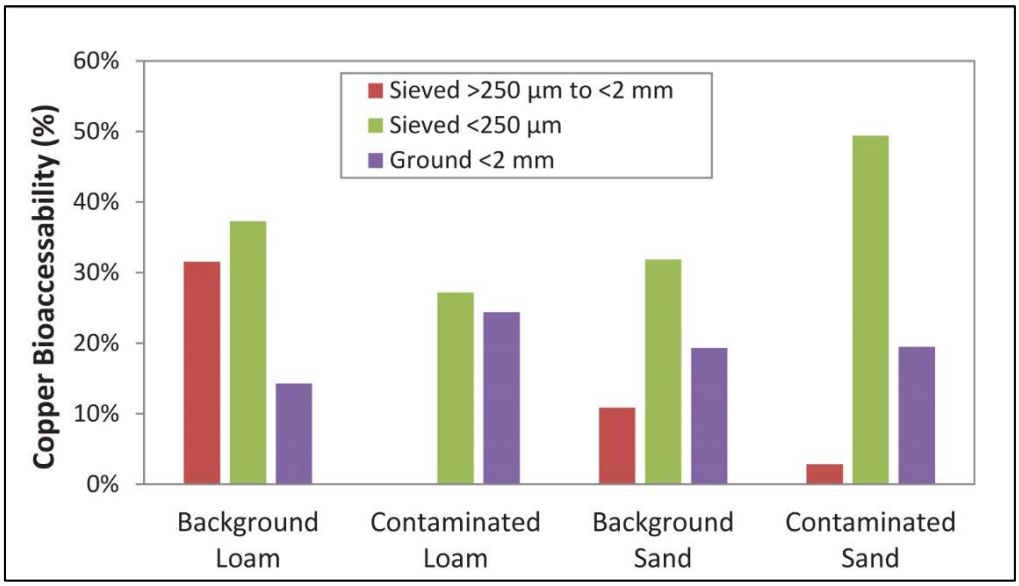


Figure 37. Physiologically based extraction technique zinc bioaccessibility.

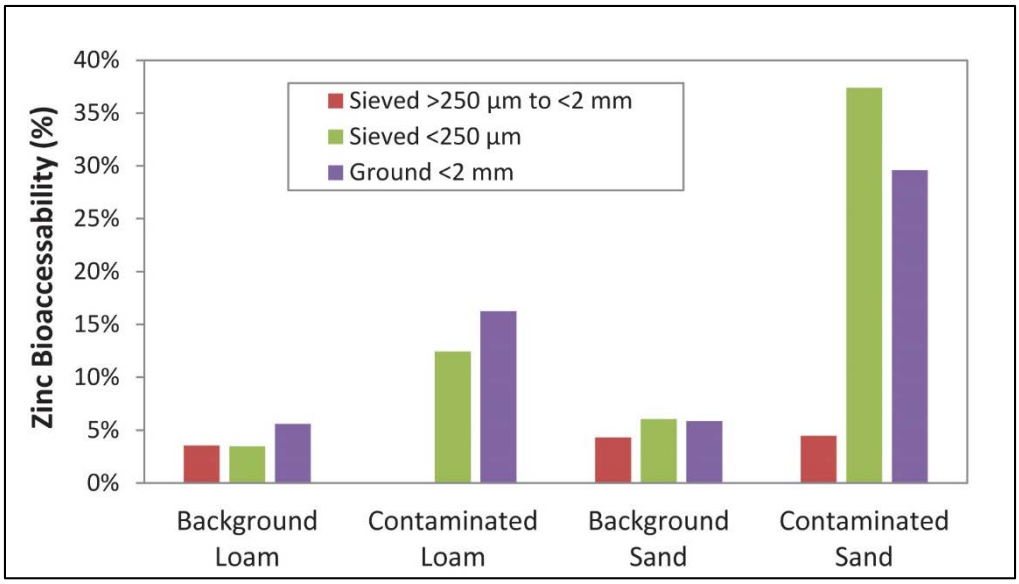


Figure 38. Physiologically based extraction technique lead bioaccessibility.

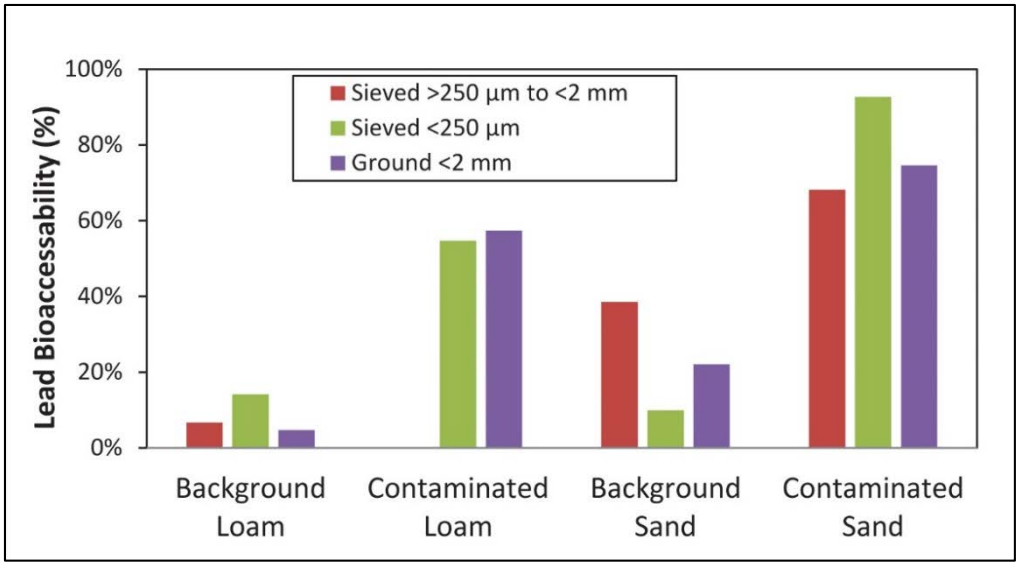
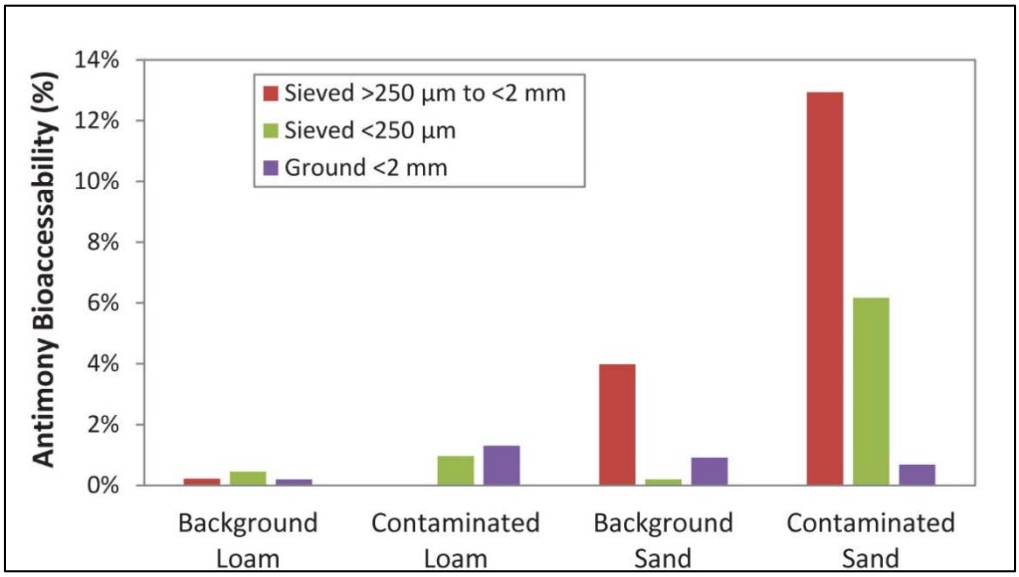


Figure 39. Physiologically based extraction technique antimony bioaccessibility.



4.3 Vegetation bioaccumulation

Figure 40 shows the uptake of lead in the roots and leaves of the ryegrass (L. rigidum) separated by soil particle size for the contaminated loam soil. In Figure 40, the leftmost first two bars represent the conventional sample preparation method, the second two bars represent a conservative approach, and the third two bars represent the ISM approach. Four replicates were conducted for each treatment. There was higher plant uptake of lead in the root tissue (oranges) versus leaf matter (green); and for both

root and leaf tissue, the greatest amount of lead uptake occurred in the <250 µm material (Figure 42). The ISM (milled) approach was intermediate between the <250 µm and the conventional approach.

In comparison, the amount of lead uptake was considerably greater for the sand by a factor of 2 to 5 times (Figure 41). However, the pattern of uptake in the sand was similar to the loam with more lead sequestered in the roots versus leaves and highest uptake in the <250 µm material.

Figure 40. Lead uptake (mg/kg) into the leaves (*green*) and roots (*brown*) of rye grass in contaminated loam.

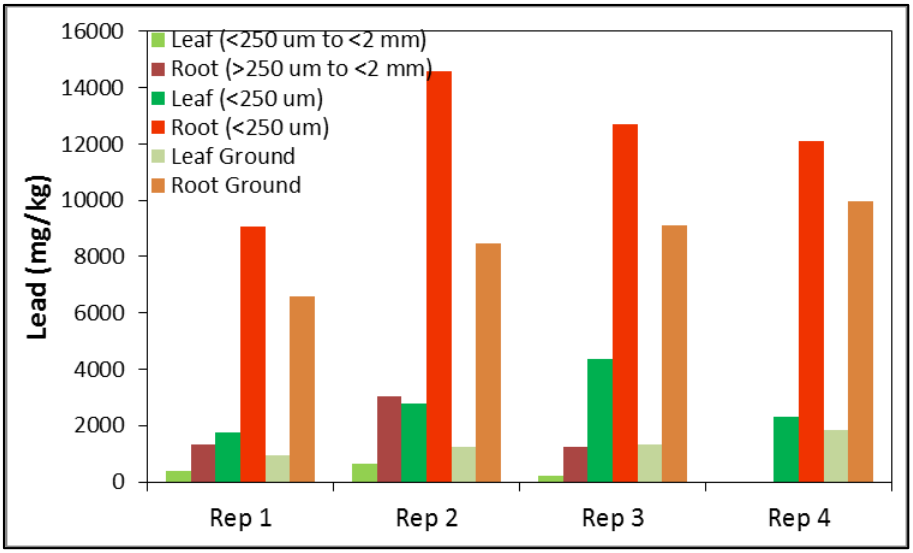


Figure 41. Lead uptake (mg/kg) into the leaves (*green*) and roots (*brown*) of rye grass in contaminated sand.

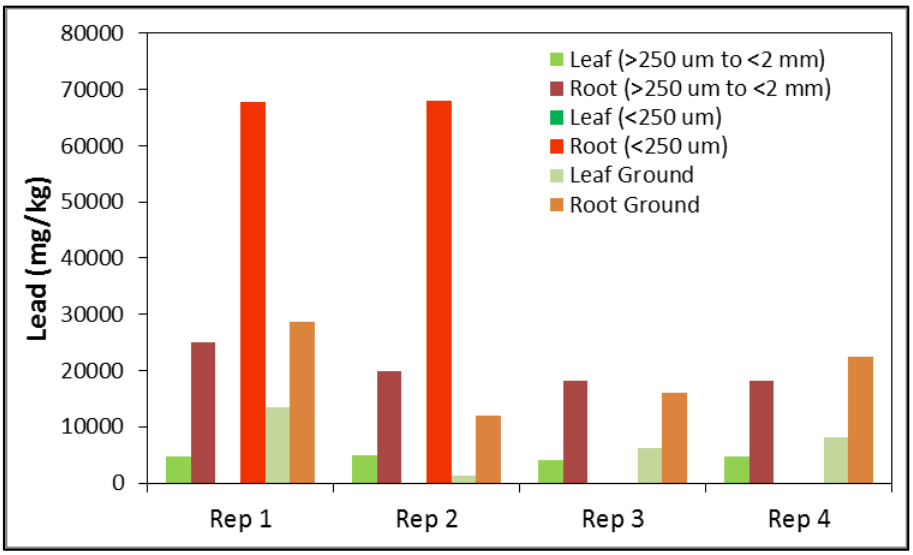
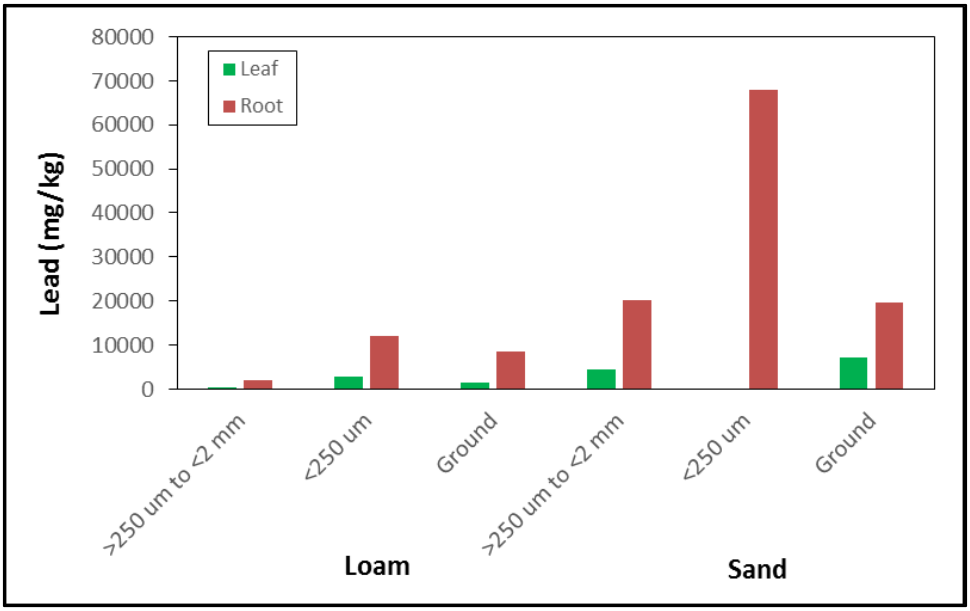


Figure 42. Average lead uptake (mg/kg) in the leaves (*green*) and roots (*brown*) of rye grass in contaminated loam and sand.



5 Discussion

The objectives outlined at the beginning of the study included (1) identifying an appropriate bioavailability test and comparing it with Method 3050, (2) assessing the impact of ISM on soil and sediment metal concentrations and its influence on the human and ecological risk assessment process, and (3) determining the appropriate disposition for the soil and sediment oversize fraction (i.e., material less than 2 mm in diameter).

5.1 Bioavailability assessment

Scatter plots show metal concentrations in the various tissue samples with the corresponding concentrations in the soil by the various digestion methods. For example, Figure 43 is a scatter plot for the concentration of lead in the worm tissue versus the corresponding soil concentration for a number of digestion methods and regression lines that pass through the origin. Note that none of the y-intercepts for the linear ordinary least squares (OLS) regression fits were significantly different from zero. Table 17 presents the slope of each regression line and the square of Pearson's r. The slope of the regression line can be viewed as a measure of bioavailability. A slope near one indicates the digestion method for the soil sample predicts the concentration in the corresponding tissue sample in an unbiased manner. A slope much smaller than one indicates the digestion method produced a concentration positively biased relative to the concentration in the corresponding tissue sample. A slope much larger than one indicates the digestion method yields a positively biased concentration relative to the tissue concentration. As shown in Table 17, the slopes for the various digestion methods for lead range over several orders of magnitude. The EDTA digestion method for lead has the smallest slope (0.01); the SPLP digestion method has the largest slope (0.90). Method 3050 for lead results in a slope of 0.07. The slope strongly depends on the nature of the digestion method and on the specific metal. A comparison of similar digestion methods for different metals extracted yields different slopes, which is evident in a comparison of Figure 43 and Figure 44 for lead and copper. Although not shown, this difference is apparent for all of the anthropogenic metals measured, included antimony and zinc, and for the native metals (e.g. Aluminum, iron, manganese, etc.).

Figure 43. Average lead uptake (mg/kg) in earthworms versus soil concentration by digestion method.

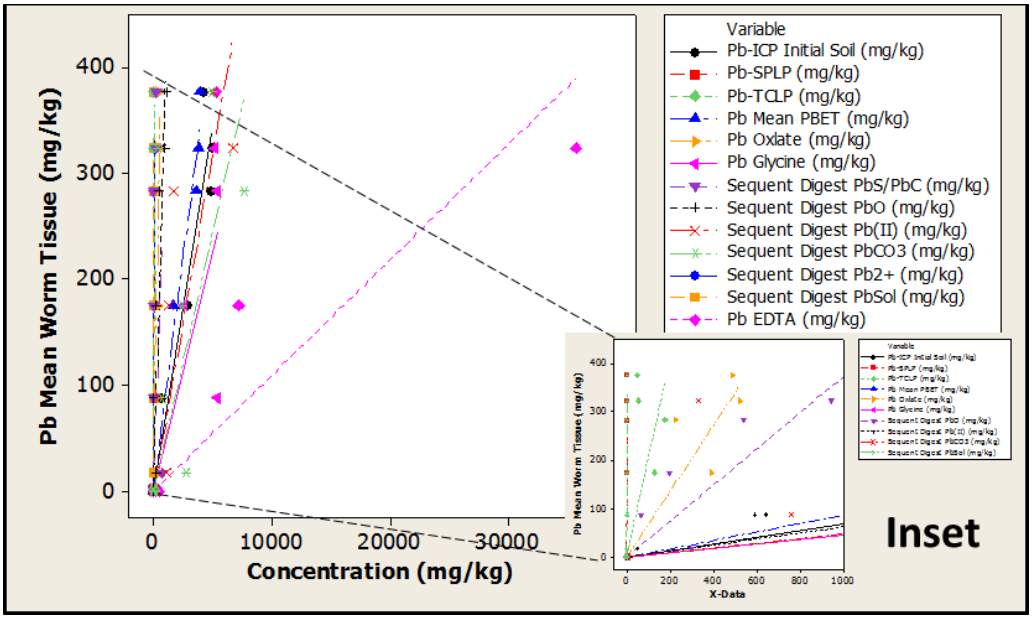
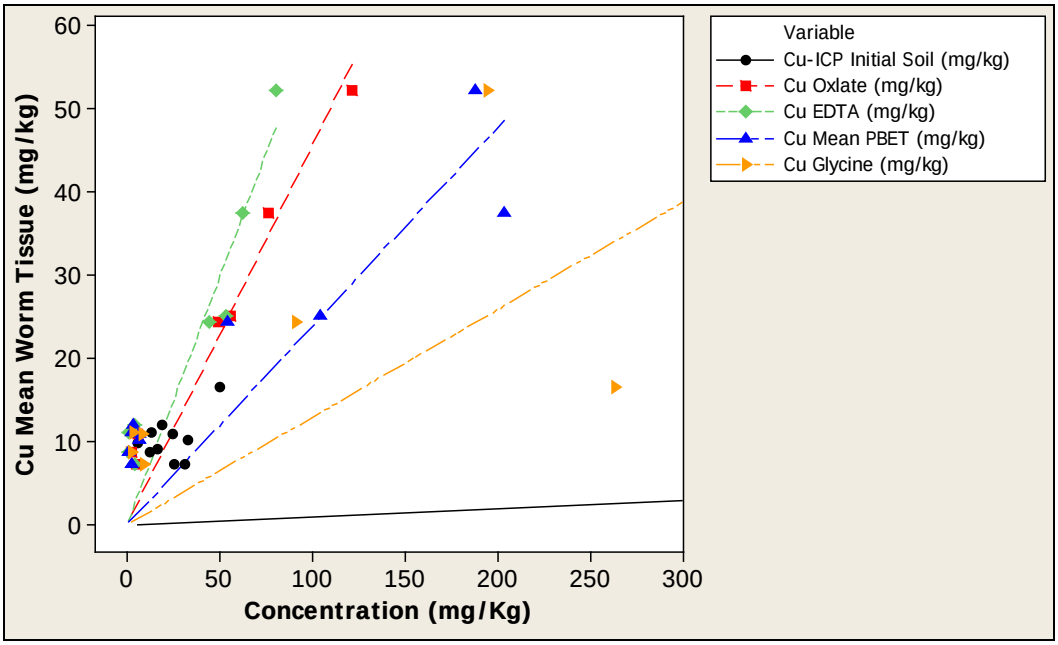


Table 17. Lead (mg/kg) worm tissue versus soil concentration.

Extraction Method/Parameter	Slope $y = m\,x$	Correlation Coefficient r^2
EDTA	0.0109	0.451
Glycine	0.0448	0.831
Sequential Digest $PbCO_3$	0.0484	0.615
Sequential Digest Pb(II)	0.0633	0.842
ICP 3050B	0.0690	0.966
PBET	0.0872	0.991
Sequential Digest PbO	0.3735	0.915
Pb Oxalate	0.6799	0.922
TCLP	2.036	0.685
Sequential Digest Pb^{2+}	4.20	0.685
Sequential Digest Soluble Pb	54.05	0.862
SPLP	85.50	0.459

Figure 44. Average copper uptake (mg/kg) in earthworms versus soil concentration by digestion method.



In addition to the type of method or digestion acid, the slope of the regression line appears to depend on the specific metal and type of sample (soil, leaf, root, and worm). As shown in Table 18, use of the same digestion method on the worm tissue samples yielded slopes for copper and lead that differ by multiplicative factors. For example, use of EDTA for soil extraction yielded a slope for copper near 1, while the slope for lead is nearer 0.01, a difference of nearly two orders of magnitude. Similarly, use of Method 3050 on the worm tissue samples resulted in a slope for copper less than 0.01 as opposed to a slope of near 0.07 for lead.

Table 18. Copper (mg/kg) worm tissue versus soil concentration.

Extraction Method/Parameter	Slope $y = mx$	r^2 $y = mx$	Slope $y = mx + b$	r^2 $y = mx + b$
EDTA	0.5927	0.938	0.04701	0.918
Glycine	0.1291	0.711	0.08279*	0.474*
ICP 3050B	0.009453	0.520	0.005810	0.436
PBET	0.2382	0.862	0.1760	0.898
Cu Oxalate	0.4553	0.954	0.3628	0.986

* Intercept is not significantly different from zero with 95% confidence.

The same digestion method produced different slopes for different types of tissue samples. For example, for the EDTA digestion method, there is no

significant correlation between lead in the soil samples and lead in either the corresponding root or leaf samples (Tables 19 and 20); but the correlation is significant for the worm tissue samples (Table 17). Similarly, for Method 3050, the slopes for lead for the root and leaf samples (Figures 45 and 46) range from about 0.7 to 4; in contrast, the slope for lead for worm tissue is about 0.007. Based on these results, the bioavailability as measured by OLS is strongly dependent on the nature of the digestion method, tissue, and metal. Consequently, the effect of milling the soil samples relative to digesting the unmilled soil samples is expected to be small if not negligible.

Table 19. Lead (mg/kg) ryegrass leaf tissue versus soil concentration.

Extraction Method/Parameter	Slope	Correlation Coefficient r^2
EDTA	No significant correlation	No significant correlation
Glycine	1.16	0.694
$PbCO_3$	0.606	0.489
Sequential Digest Pb(II)	No significant correlation	No significant correlation
ICP 3050B	0.668	0.425
PBET	1.292	0.599
Sequential Digest PbO	No significant correlation	No significant correlation
Pb Oxalate	No significant correlation	No significant correlation
TCLP	48.3	0.956
Sequential Digest Pb^{2+}	No significant correlation	No significant correlation
Sequential Digest Soluble Pb	No significant correlation	No significant correlation
SPLP	2193	0.894

Table 20. Lead (mg/kg) ryegrass root tissue versus soil concentration.

Extraction Method/Parameter	Slope	Correlation Coefficient r^2
EDTA	No significant correlation	No significant correlation
Glycine	3.201	0.742
$PbCO_3$	2.367	0.877
Sequential Digest Pb(II)	No significant correlation	No significant correlation
ICP 3050B	4.007	0.764
PBET	5.555	0.921
Sequential Digest PbO	17.7	0.476
Pb Oxalate	31.5	0.700
TCLP	133.8	0.939
Sequential Digest Pb^{2+}	No significant correlation	No significant correlation
Sequential Digest Soluble Pb	4214	0.336
SPLP	6170	0.905

Figure 45. Average lead uptake (mg/kg) in ryegrass leaf tissue versus soil lead by digestion method.

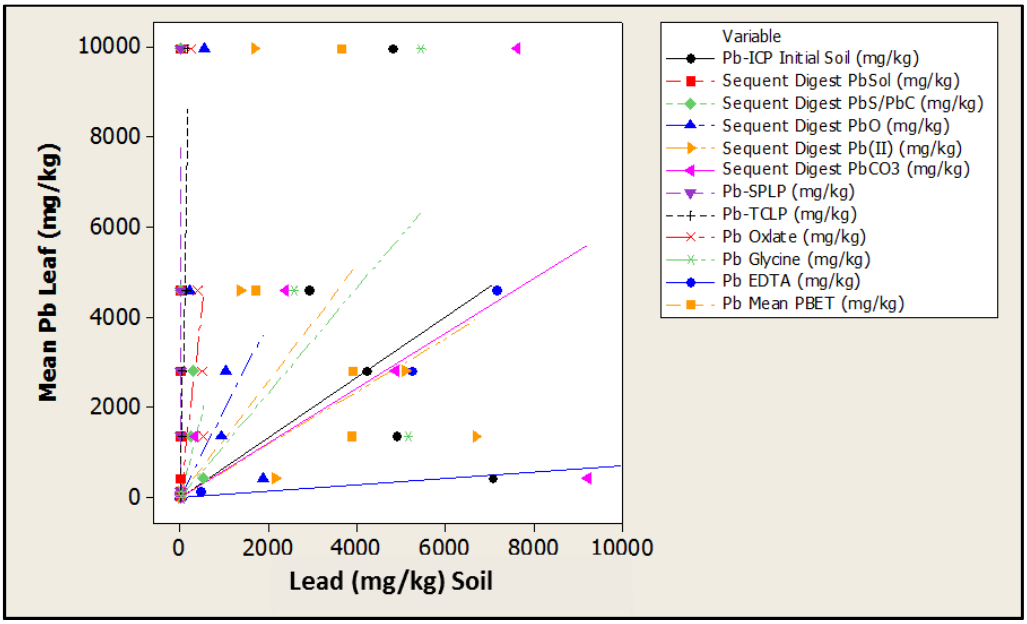
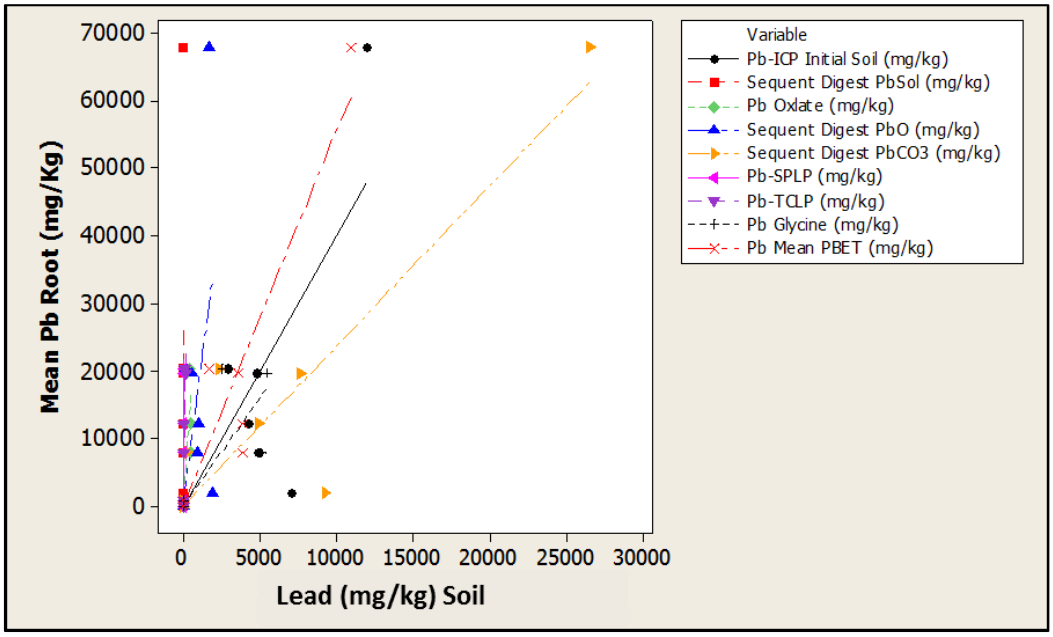


Figure 46. Average lead uptake (mg/kg) in ryegrass root tissue versus soil lead by digestion method.



5.2 Incremental sampling methodology impact on metal bioavailability

The same digestion method produced different slopes for different types of tissue samples. For example, for the EDTA digestion method, there is no significant correlation between Pb in the soil samples and Pb in either the corresponding root or leaf samples; but the correlation is significant for the worm tissue samples. Similarly, for Method 3050B, the slopes for Pb for the root and leaf samples range from about 0.4 to 4; in contrast the slope for Pb for worm tissue is about 0.007. Based on these results, the bioavailability as measured by OLS slopes strongly depends on the nature of the digestion method, tissue, and metal. Relative to these factors, the effect of grinding the soil samples relative to digesting the soil samples unground is expected to be small if not negligible.

To test the hypothesis that milling does not greatly affect inferences about bioavailability, the study evaluated the biota and animal tissue samples exposed to milled and unmilled soils. The samples were split into milled and unmilled aliquots. The milled soil was sieved through a 2 mm sieve prior to milling, and the unmilled aliquots were additionally fractionated by sieving. Each unmilled soil was divided into three fractions via sieving: >2 mm, 0.25 to 2 mm, and <0.25 mm. The mass of the last two fractions are denoted by m_1 and m_2, respectively. Therefore, to compare the milled and unmilled soils for the two <2 mm fractions, it was necessary to apply mass weighing factors to these two mass fractions:

$$w_1 = m_1 / (m_1 + m_2) \quad (4)$$

$$w_2 = m_2 / (m_1 + m_2) \quad (5)$$

where

w_1 = the weighting factor for the 0.25 to 2 mm size fraction,
w_2 = the weighting factor for the <0.25 mm size fraction,
m_1 = the mass of the 0.25 to 2 mm size fraction, and
m_2 = the mass of the <0.25 size fraction.

The mass-weighted concentrations of Pb in the unground soils were very similar to the concentrations of Pb in the ground soils (Table 21).

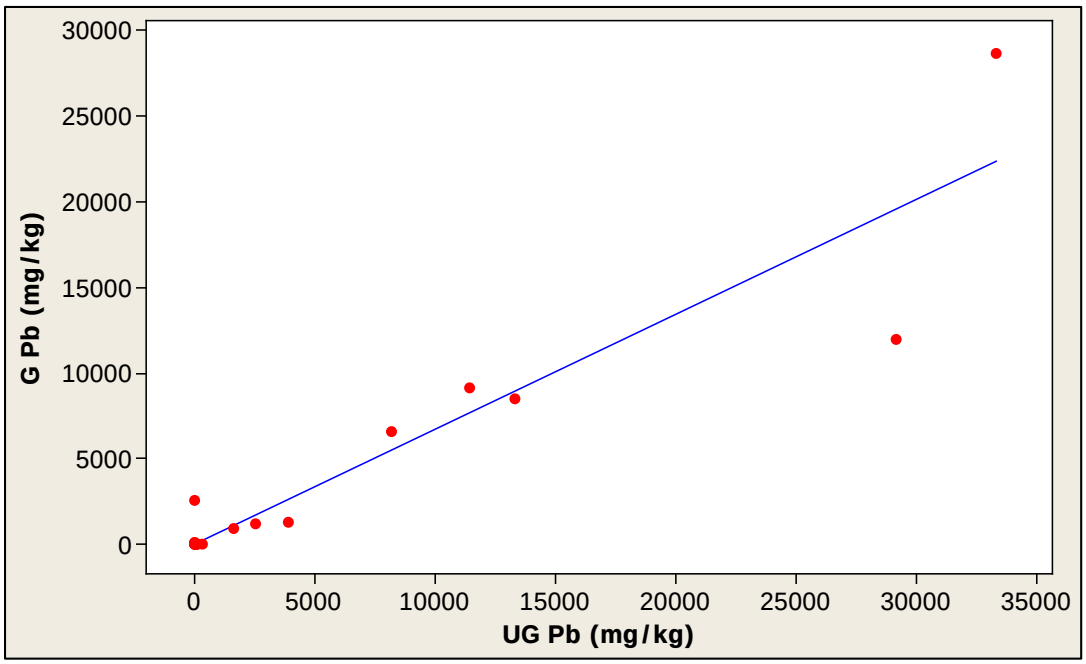
Table 21. Lead concentration by soil type and processing method.

Soil Type	Lead (mg/kg)
UG Background Loam	11.63
G Background Loam	11.50
UG Contaminated Loam	4566.96
G Contaminated Loam	4900.00
UG Background Sand	5.83
G Background Sand	3.55
UG Contaminated Sand	4680.51
G Contaminated Sand	4820.00

G = ground, UG = unground

For the unmilled soils, these weighting factors were applied to the corresponding tissue concentrations. The ground tissue results were subsequently compared with the weighted unmilled tissue results. Figure 47 indicates the differences between the milled and unmilled (G and UG, respectively) tissue lead concentrations were small with Pearson's and Spearman's correlation coefficient of 0.94 and 0.62, with $p < 0.003$. The Wilcoxon test indicated that the median of the differences was significantly less than zero with over 99% confidence. Though the residuals were not normally distributed, an OLS regression fit forced through gave the equation milled = 0.7 × unmilled, suggesting the milled results are negatively biased relative to the unmilled results. It is not clear why the milled results should be negative biased relative to the unmilled results or whether the result is of practical significance. However, the result of the evaluation (e.g., a slope near one) suggests that relative to factors such as metal type, tissue type, and method of digestion, inferences about bioavailability will not strongly depend on whether the soils are milled prior to digestion.

Figure 47. Milled versus unmilled lead (mg/kg) tissue levels.



5.3 Oversize fraction disposition

Our study compared the metals (antimony, copper, lead, and zinc) mass for the different soil particle sizes by soil type and by background and contaminated material (Table 22). In the contaminated loam, copper and lead were predominant in the <0.25 mm fraction. In contrast, antimony was predominant in the >2 mm fraction and zinc in the 0.25 to 2 mm fraction. The distribution of metal was entirely different in the contaminated sand with copper and zinc dominant in the >2 mm fraction and antimony and lead the primary metals in the 0.25 to 2 mm fraction. Depending on the metal of interest and the type of soil, the oversize fraction (>2 mm) could be an important contributor to the total metal mass.

The current ecological and human risk calculation method generally relies on the use of a soil concentration. That analysis considers material <2 mm in size or in some case <0.25 mm only. Many risk assessors rely on the <0.25 mm fraction for calculating risk of lead as material in this size class has the potential to stick to a hand due to electrostatic properties. Consequently, children playing in lead-contaminated soil could have dermal exposure and could ingest contaminated lead by licking their hands, resulting in internal exposure. The risk community and USEPA often view the use of <0.25 mm as resulting in the most conservative risk calculation. The

>2 mm material is not considered soil by USEPA definition and consequently is not considered in risk calculations. Because a larger particle size equates to a smaller surface area available for dissolution, the amount of bioavailable metal released into an aqueous solution is rate limited. However, our previous work with tungsten solid residues on small-arms ranges indicated a significant portion was available in the >2 mm fraction (Clausen et al. 2007). Consequently, a general rule pertaining to the availability of metal in the >2 mm fraction appears to be metal and matrix dependent. Further, results in Table 22 suggest that risk calculations based solely on the <0.25 mm soil size fraction do not necessarily yield the most conservative risk value.

Because a sizable portion of the total metal mass can reside in the oversize fraction, our recommendation is that the concentration of metal in the oversize fraction should be determined. The concentration information should then be converted to mass units and added to the calculated mass for the <2 mm fraction. The mass information can then be back converted to a metal concentration for the entire sample collected. The current practice of relying on the <0.25 mm fraction to yield the most conservative lead risk values is based on an erroneous assumption that the majority of lead resides in this size class. It is also clear that the other metals are not necessarily predominant in the smallest particle size fraction.

Table 22. Computed metal mass by soil particle size.

Particle Size	Soil Mass (g)	Sb Mass (g)	Sb (mg/kg)	Sb (%)	Cu Mass (g)	Cu (mg/kg)	Cu (%)	Pb Mass (g)	Pb (mg/kg)	Pb (%)	Zn Mass (g)	Zn (mg/kg)	Zn (%)
Background Loam													
Total Sample	4.39	NA		NA	0.12		100.00	0.05		100.00	0.17		100.00
>2 mm	0.61	NA	<1.00	NA	0.02	33.0	16.65	0.00	15.4	5.36	0.02	30.9	11.24
250 um to 2 mm	0.46	NA	<1.00	NA	0.02	24.5	13.11	0.01	11.1	15.26	0.02	37.1	10.18
<250 µm	3.32	NA	<1.00	NA	0.08	26.0	70.24	0.04	11.5	79.38	0.13	39.7	78.59
Contaminated Loam													
Total Sample	3.96	0.05		100.00	1.31		100.00	13.07		100.00	1.64		100.00
>2 mm	1.11	0.04	0.00	82.53	0.01	0.00	0.50	0.06	7080	0.43	0.02	4360	1.08
250 µm to 2 mm	0.32	0.00	29.3	0.00	0.00	517	0.00	2.27	4250	17.33	1.40	90.8	84.93
<250 µm	2.53	0.01	13.8	17.47	1.31	4270	99.50	10.75	4900	82.24	0.23	510	13.98
Background Sand													
Total Sample	10.66	NA		NA	0.17		100.00	0.04		100.00	0.18		100.00
>2 mm	6.64	NA	<1.00	NA	0.11	12.3	67.30	0.01	3.65	38.54	0.09	20.3	49.56
250 µm to 2 mm	3.29	NA	<1.00	NA	0.04	18.8	24.42	0.01	15.6	31.54	0.07	29.8	38.05
<250 µm	0.73	NA	<1.00	NA	0.01	13.7	8.28	0.01	3.55	29.91	0.02	22.7	12.39
Contaminated Sand													
Total Sample	7.74	0.82		100.00	53.84		100.00	19750		100.00	6.46		100.00
>2 mm	4.42	0.16	16.6	19.51	39.6	4100	73.49	2930	28.3	14.84	4.69	486	72.61
250 µm to 2 mm	2.68	0.62	141	75.92	10.1	2290	18.80	12000	53.0	60.76	1.25	282	19.30
<250 µm	0.64	0.04	14.0	4.57	4.15	1550	7.72	4820	12.9	24.41	0.52	195	8.09

6 Conclusion

The highest lead levels were in the unmilled <0.25 mm material for both loam and sand whereas the milled <2 mm soil material yielded metal levels 20% to 40% lower than unmilled <0.25 mm material but higher than the 0.25 to 2 mm material. However, these observations were not consistent for all of the anthropogenic metals.

Lead speciation depended on soil type and contamination (natural versus anthropogenic), but significant differences were not evident by particle size or the milled versus unmilled soil. Lead carbonate was the dominate species for contaminated soil, followed by Pb(II), whereas lead oxides dominated in the uncontaminated soil. In the uncontaminated soil, the Pb^{2+} ion was dominant.

Plant uptake of lead for the ryegrass (*L. rigidum*) was highest in the roots as compared to leaf tissue. Lead fluxes in the ryegrass studies were highest in the unmilled <0.25 mm sand material. The highest earthworm (*E. fetida)* 14-day mortality (100%) was for earthworms exposed to the <0.25 mm sand. In contrast, earthworm 14-day mortality was less than 20% for the unmilled <0.25 mm clay material. Not surprisingly, a relationship is evident between lead soil concentrations and lead worm-tissue concentrations.

The *L. rigidum* and *E. fetida* in vivo experiments and digestion studies indicate that basing a risk assessment on the results from unmilled <0.25 mm material may overestimate the true risk. A good compromise between risk overestimation with <0.25 mm material and risk underestimation with <2 mm material is results obtained with milled soil following ISM. In conclusion, application of the ISM as outlined in the upcoming USEPA Method 3050C provides the best estimate of risk for metals when present in soils as particulates.

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14. ABSTRACT

This study assessed the impact of the incremental sampling methodology (ISM) on metals bioavailability through a series of digestion and in vivo experiments. These tests used *Eisenia fetida* and *Lolium rigidum* in both milled and unmilled loam and sand soil containing antimony, copper, lead, and zinc obtained from Donnelly Training Area, Alaska. No significant differences in metal levels were evident between milled and unmilled soil for *E. fetida*, and uptake of lead by *L. rigidum* in sand yielded lead recoveries comparable with Method 3050 analysis of soil. In contrast, *L. rigidum* grown in loam had much lower recoverable lead. Milling of the soil as part of the ISM process had no significant impact on the lead species distribution. In comparison with Method 3050, the alternative digestion tests involving the use of glycine; oxalate; ethylenediaminetetraacetic acid (EDTA); or alternative digestion procedures, such as the synthetic precipitation leaching procedure (SPLP) and the toxicity characteristic leaching procedure (TCLP), yielded lower recoveries of lead for all soil particle sizes and soil types. Diffusive gradient in thin films experiments yielded metal concentrations positively correlated with *E. fetida* concentrations. The physiologically based extraction technique (PBET) positively correlated with bulk soil concentrations and *E. fetida* tissue concentrations for all soils evaluated.

15. SUBJECT TERMS

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